

Deciding about embryo research

Four years late, the British government has published a bill to regulate embryo research. The need now is that the British parliament should take an enlightened view of the freedom of research.

THE British government's Embryo Bill (published last week) may be much as advertised, but the emergence of the Royal Society as a potentially stout lobbyist on some of the issues raised is unexpected. What the bill proposes is what the Warnock report recommended — all procedures involving the *in vitro* fertilization of human eggs by human sperm will have to be licensed by an authority yet to be created, so that unlicensed procedures will be criminal offences. So much is uncontroversial. The bill would also regulate the practice of artificial insemination of women, requiring that information about the contributors to sperm-banks should be kept so that it should be possible to determine the genetic relationships of intending marriage partners.

What will most exercise the British parliament in the next few months is the single issue of whether researchers should have 14 days in which to investigate the behaviour and properties of new embryos following protocols which have themselves been licensed. The article by Dr Anne McLaren on page 469 of this issue forcefully explains why members of parliament should follow the Warnock recommendation. The hazard is the government's decision that this issue should be considered a matter of "conscience" on which members of the House of Commons (MPs) will not be bound by the customary party discipline. The months ahead would be more comfortable for British science if were clearer that the consciences of MPs would be troubled if constructive and potentially valuable research were forbidden.

This is the point on which the Royal Society plans to intervene, and rightly. Already it has held one meeting with the House of Lords to explain why it believes that research with embryos should continue, but under licence. One formal statement put out last week follows the standard lobbyists' practice of urging its readers to write to their MPs. Another is a succinct "fact-sheet" whose chief influence will be that of an excellent diagram showing the early stages in the development of the human conceptus, but which also significantly lists the benefits of research with human embryos even beyond the 14-day limit specified by Warnock.

The Royal Society's welcome intervention in this public debate may be unprecedented. On many previous occasions, the society has held that the diversity of its membership prevents it making statements beginning, "The Royal Society believes . . ." (in this case, that "it is

essential that research, under proper control, should be allowed to continue"). When the dust has settled on the Embryo Bill, maybe the society will turn its attention to the parlous condition of British science as a whole.

This journal's opinion is that the 14-day Warnock limit is neither necessary nor wise (see *Nature* **341**, 673, 26 October, 1989). It is not necessary because the bill would give the government the power to regulate the conditions under which the "Human Fertilization and Embryology Authority" grants licences. Why not start with a 14-day limit imposed by regulation with the intention of relaxing that if the new authority can make a convincing case for doing so, and win public acceptance for its plans? Legislation with a formal time limit built in will be much more difficult to amend.

Defects

The other defects of the bill may be less important, but deserve attention. The composition of the proposed authority is one of them. The intention is that it should be a "body corporate" without the protection of crown privilege raising funds either from licence fees or from the public purse. The chairman and deputy chairman will be neither physicians nor people with a vested interest in the procedures and research to be regulated, but the bill says that "at least one-third but fewer than half" of the other members should be such people. That argues for at least five other members (two physicians and three others), but the otherwise sensible appeals procedure proposed requires that nobody taking part in a disputed decision should take part in its reconsideration, which seems to imply that the minimum size of the authority is multiplied by two. That sounds cumbersome, to say the least. And it is strange that an authority whose success will hang on its capacity to win public confidence should be required to report only every other year.

The proposed databank of genetically relevant information raises other problems. It is sensible, even essential, that there should be means by which people who believe or even suspect that they are the offspring of a regulated procedure can discover whether that is the case, and in particular discover that they are not genetically related to, say, an intended spouse. So the authority's databank will contain sensitive and potentially damaging information. But the bill proposes that people who improperly disclose that information should be liable to



up to two years in prison, while those guilty of breaching the interdictions on practice and research should be liable to ten years in prison. Because the authority's work must command public confidence, the penalty for breaching confidentiality should be greater.

These and other issues require careful consideration in the months ahead. Let us hope that they are not too much confused by the side-issue of abortion. The Royal Society (among others) hopes that the issues can be kept quite separate, but is likely to be disappointed. For one thing, the implication that embryos older than 14 days deserve special respect is certain to fire the ambitions of those who believe that much older fetuses are at present held in scant regard. For another, because one of the most cogent arguments for allowing embryo research is that of diagnosing genetic defects before the transfer of embryos to a uterus, so avoiding abortions at a later stage, those who hold to the extreme position in anti-abortion doctrine that no interference with the lottery of human reproduction is allowable can hardly be completely silenced. □

Priority on HIV

A US newspaper has raised questions about the discovery of the virus responsible for AIDS that compel explanation.

WHO first discovered the virus responsible for AIDS which is now called HIV (for human immunodeficiency virus)? At least in the United States, most people would answer "Dr Robert Gallo", referring to the head of the US National Cancer Institute's Laboratory of Tumor Cell Biology. Others, of course, will give a more sophisticated reply, saying "Gallo and Montagnier" — the second a reference to the researcher at the Institut Pasteur in Paris who is known, among many other things, as the inventor of the diagnostic test for HIV over which the Pasteur launched a patent suit against the US government in 1985. The case was eventually settled out of court in April 1987 by an agreement between the then US President Ronald Reagan and the then French prime minister, Jacques Chirac, who agreed that the battle against AIDS is too important to be marred by disputes over patents, and who decided (among other things) to use part of the royalties in dispute for supporting research in the field.

Yet the acrimony generated by that dispute and its antecedents is curiously persistent. Its latest manifestations is the appearance in the *Chicago Tribune* for 19 November of a gripping sixteen-page report by journalist John Crewdson of his investigations of the circumstances leading to the recognition that HIV is the cause of AIDS. Crewdson has discovered and recounts the circumstances leading to the various publications by Gallo and Montagnier in the period up to 1987 which, so far as they affect this journal directly, are correct. (He might nevertheless have told his readers what he had been told explicitly — that the decision not to publish Montagnier's nucleotide sequence of HIV as well as Gallo's, which arrived first,

was prompted by a wish not to publish the same sequence twice. Ironically, for *Nature*, the similarity has fuelled much of the later controversy.) The general tenor of his report is that Gallo has been given too much credit, and Montagnier too little.

Gallo and his associates will not be amused. Gallo may at this stage kick himself for not having collaborated with Crewdson after a first telephone conversation, so allowing what may be a one-sided account of various incidents to colour the report. It is always difficult for professional people to judge how much time — it does take endless time — they should invest in answering the questions of investigative reporters. Gallo's decision may well have been influenced by the agreement already reached between the French and the US governments, which included a chronology of the events leading to the identification of HIV drawn up jointly by him and Montagnier and an agreement that neither would further comment on past history. Did that not bury the priority dispute? he may well have said.

Sadly, Crewdson's report is forceful, or at least prominent, enough to resurrect the controversy, which is damaging not only for the reputation of science but for the chief participants, Montagnier as well as Gallo. Yet the report is at once so wide-ranging and so particular that nobody could easily respond to it. Gallo, the one most directly attacked, would nevertheless do well to say something by way of refutation, most tellingly by dealing with some of the particular points that Crewdson raises. There is, for example, the letter from Gallo and several of his associates published in *Nature* in May 1986 (not April, as Crewdson avers) which shows an electron micrograph containing now unmistakable HIV particles budding from the white blood cells of a sample provided (from a Paris hospital) in February 1983 (see *Nature*, **321**, 119; 1986). The burden of the letter, headed "First isolation of HTLV-III" (Gallo's name, chosen later, for HIV), was that a novel virus had been recognized at the National Cancer Institute at least by the first half of 1983, and specifically before Montagnier's first sample reached Bethesda in September that year. But Crewdson claims that Gallo did not recognize the presence of a novel virus until much later. Gallo says that Crewdson's account is incorrect, giving chapter and verse for his belief. Luckily, with outside collaborators involved, it is an issue that can be checked. It would be helpful if it were.

Montagnier also has a part to play, if only as a party to the agreed chronology of the events. Crewdson's meanest charge is that the two men have struck a truce so as the better to be able to share a Nobel prize one of these days. If that were so, it would be disgraceful, but the charge cannot be convincingly denied. Even a joint declaration of innocence could be interpreted as proof positive. Yet both must know by now that between 1983 and 1985 they were engaged in one of the most exciting and successful discoveries of this decade. Cannot they jointly find a way of making sure that this episode is not soured by a priority dispute waged by others than themselves? □

Human genome project a cause of friction

- James Watson's warnings resented in Japan
- Space station and SSC also controversial

Tokyo

RESEARCHERS trying to promote the human genome project in Japan are dismayed by recent suggestions by James Watson, director of the US National Institutes of Health Center for Genome Research, that countries failing to contribute financially to the project should be denied access to US genome data. The remarks exacerbate growing resentment among Japanese scientists at US pressure to join big US-backed projects.

Watson made the comments at a congressional hearing in October (see *Nature* **341**, 679; 1989). And a few months earlier, he privately told Kenichi Matsubara, Japan's representative for the Human Genome Organization (HUGO), that Japan should not expect to benefit from the genome activities of other nations if it does not contribute to HUGO on a scale commensurate with Japan's economic stature.

His remarks are being taken seriously in Japan. In October, Itaru Watanabe, vice-president of the Science Council of Japan, an elected body of 210 scientists, at a meeting of the council to promote the project, warned that "if Japan alone does not contribute to research in this field, Japanese scientists will be blocked from access to overseas information".

Matsubara says that Japanese scientists "fully support" Watson's well-publicized sentiment that Japan as a prosperous nation should contribute more to basic science. But they are perplexed by his specific request for money for HUGO, coming as it does at a time when HUGO is just about to discuss details of its activities and budget. Without such details, it is hard for Japanese scientists to raise funds.

Furthermore, under Japanese law, the Japanese government cannot contribute

money directly to HUGO and donations from the private sector cannot be tax-deductible. Nevertheless, a newly formed task force of the Ministry of Education, Science and Culture headed by Matsubara is looking into "every possible avenue" to raise the few hundred thousand dollars required each year for HUGO. But HUGO itself must make "considerable efforts to shape up so that it can enjoy maximum support from countries with different social structures and regulations", Matsubara says.

Matsubara takes Watson's comments as an "expression of US anxiety" about Japan's genome efforts in general and a reaction to "incoherent and fragmentary information" in the United States about what is going on in Japan. Matsubara points out that various government agencies and ministries are spending more than \$10 million a year on genome-related projects. Although this is small compared with the investment in the United States, it is similar to that of several European nations. And by 1991 Matsubara is confident that the Ministry of Education's task force will have gained extra support.

But it is Watson's suggestion that countries failing to contribute to the project should be denied access to US genome data that has caused most upset. Matsubara says that such action would be "tremendously unfortunate not only for us but also for the progress of science". Japanese scientists would be "extremely annoyed by such hasty action" which would be "unprecedented in the history of international cooperation in science".

Japanese scientists and government officials are getting tired of US pressure to cooperate in big science projects. Other examples are the US Superconducting Super Collider (SSC) and the US Space Station. Having won Japanese support for the space station, the US government has now caused considerable upset in Japan (and Europe) by delaying the project and scaling down the power specifications for the station (see *Nature* **341**, 3; 1989). And in July, a Tokyo University physicist at a public lecture on the future of science indignantly asked of the SSC "why should we contribute to Texan pork-barrel politics?"

In a similar vein, Matsubara says that Japanese scientists feel that the human genome project is not something they should be forced to cooperate in "simply because the US government has decided to initiate and allocate a budget for it".

David Swinbanks

EMBRYO RESEARCH

British debate under way

London

THE British government's draft legislation to regulate the practice of *in vitro* fertilization (IVF), artificial insemination (AID) and embryo research, published last week, closely follows the recommendations of the Warnock report, published in 1984, but offers ingenious legal solutions for some of the problems raised therein.

The bill will first be debated in the House of Lords beginning in the next few weeks. As recommended by Warnock and promised by the government's white paper earlier this year, there will be a statutory authority, called the Human Fertilization and Embryology Authority, to license activities and research not permanently forbidden.

The latter include the keeping of embryos after the appearance of the primitive streak (the rudiments of the nervous system) at roughly 14 days, the transgenic transfer of human embryos and the replacement of the nucleus of one cell in an embryo by a cell from another embryo or from any other person (which is a possible route to the cloning of people).

As promised last year, Parliament will have to choose whether to allow research on human embryos between conception and the appearance of the primitive streak or to forbid it. The government says that it will not make use of party discipline to persuade its supporters vote in any particular direction.

The bill deals with surprising ease with some of the legal uncertainties raised by Warnock, notably the parentage of children born by IVF and AID. In all cases, a child's mother is defined as the woman in whose uterus it was brought to term, so that surrogacy will require formal adoption. Similarly, the father will be the mother's male spouse provided that he has consented to the procedure. Third parties who donate sperm for either IVF or AID will not be counted as fathers if they have consented to the procedures. The authority will be required to keep data allowing genetic relationships between potential marriage partners to be reconstructed.

The Royal Society has entered the forthcoming debate as a lobbyist, urging in a statement put out last week that embryo research should be allowed. The Voluntary Licensing Authority sponsored jointly by the Medical Research Council and the Royal College of Obstetricians and Gynaecologists which has changed its name to the Interim Licensing Authority, in a document of embryo research published with its annual report this week, echoes the Royal Society in its hope that the forthcoming debate will not be obscured by the abortion issue. □

Frontiersman Gowans



SIR James Gowans, formerly secretary of the UK Medical Research Council, is to be the first secretary-general of Japan's Human Frontiers Science Programme, which opened its office in Strasbourg last week (see *Nature* **342**, 334; 1989).

Slow road to cooperation

Munich

DESPITE the dramatic political changes in East Germany and the opening of the borders on 9 November, the widely expected increase in industrial and scientific contacts between the two German states is only slowly coming into view.

The West German Research Ministry (BMFT) announced last week that it expects there to be many more cooperative projects between the two German states. Perhaps as many as 20 new projects might be initiated by early next year, nearly a doubling of the current number. But financial and political hurdles must be overcome before such cooperation can flourish.

The new projects, ranging from surface science to data processing to continental deep drilling, were discussed at a meeting in October between representatives of the two states. Officials will meet early in 1990 to discuss the proposals put forward both by East Germany and by West German universities.

Ministry officials were especially optimistic that the Fraunhofer Institutes, which do applied research under contract to industry, might receive contracts from East German industrial combines.

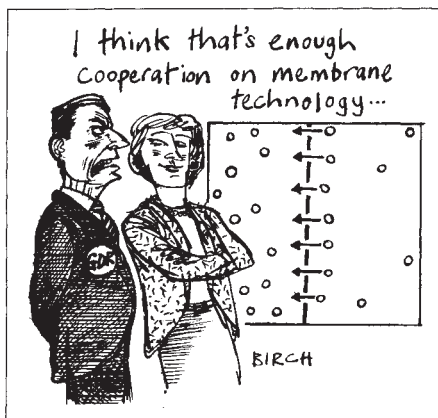
BMFT officials say their hopes of brushing aside political obstacles to cooperation were strengthened by the October meeting, which took place in Wiesbaden, West Germany, the day after East German leader Erich Honecker was deposed.

The East German officials recognized the need for economic reform in their country and wanted to explore ways to use West German knowhow to achieve it, a spokesman for BMFT said. The previous tendency to put a speed limit on cooperation was no longer there, he said.

But contacts continue to be limited by East Germany's lack of money. In many cases, the inefficiency and poor management at many combines and state-run agencies is only now coming to light. Research contacts may have to take a back seat during the many years of rebuilding necessary. Until then, East Germany will look to the West for help.

Until now, BMFT has followed the West German government line of waiting for proof of political and economic reforms — including free elections and the introduction of a market-oriented economy — before volunteering Western money for East Germany. The spokesman said the planned visit of Chancellor Helmut Kohl to East Germany in mid-December is expected to clarify the situation.

In the meantime, other political and bureaucratic problems remain. Cooperation between the two German states has always been hindered by the status of



West Berlin, which East Germany refuses to recognize as a *de facto* part of West Germany. West Berlin is still officially administered by the Western Allies. West Germany insisted on the inclusion of West Berlin researchers in all cooperation agreements, which made negotiations difficult.

Technology transfer to East Germany could also be held up by the CoCom restrictions on high-technology exports, which remain in force despite the political changes in the Soviet bloc. The current agreement on scientific cooperation between the two German states, signed in Bonn in September 1987, includes few projects that involve high technology (see *Nature* 329, 192; 1987). **Steven Dickman**

MAX PLANCK SOCIETY

Expanding contacts

Munich

THE Max Planck Society expects to expand its contacts with the East German Academy of Sciences beginning early next year.

During a visit of academy president Werner Scheler to Munich in mid-October, the two organizations agreed to work out a 'framework agreement' for future links between their organizations. The agreement would make it easier for researchers on one side to invite and collaborate with their counterparts in the other country and would lay down the financial terms for the visits.

A spokesman for the Max Planck Society said that of 384 official guests last year from the Soviet bloc and China, not a single one came from East Germany.

The Max Planck Society has 62 institutes and an annual budget of DM 1,240 million (\$670 million). It has 13,000 employees, who do primarily basic research. The Academy of Sciences of the German Democratic Republic, modelled on the Soviet Academy of Sciences, has 70 institutes and 23,000 employees. Its annual budget of 1,000 million East German marks is spent both on basic research and applied research for industrial combines (*Kombinate*).

Steven Dickman

Research still a priority

Berlin

STREET demonstrations, shouted slogans, and the triumphant return *en masse* of East Germans to West Berlin after a 28-year absence give the impression that life in East Germany is one big party. It only adds to the elation that the East German government appeared so receptive to the changes proposed by opposition groups.

But the party is coming to an end, as the opposition, led by New Forum, must make its own proposals for the construction of a new society on the ruins of the old.

The state of the East German economy is more worrying than is often supposed. Much of East German industry faces collapse after decades of neglect. Even such staples as energy for home heating could be interrupted, with serious consequences.

Against this backdrop, the future of research institutes may seem trivial, but New Forum members are convinced that research, both basic and applied, is central to the future of the economy. If research is not protected, they warn, "the next crisis is already programmed".

New Forum emerged earlier this year when the 18-year rule of Erich Honecker was seen to be running out of time. "A change was in the air", says physicist Sebastian Pflugbeil, one of the few dozen founders.

At the outset banned, New Forum has become one of the most popular political groups in East Germany, although it clings to its identity as a "citizens' initiative" and has resisted pressure to become a political party. It has been the catalyst for many of the street demonstrations of recent weeks.

New Forum is not so much interested in reforms within the government party as with the rot that reaches much further down in all areas of society, including science. "Too many department heads have prevented their subordinates from doing effective research work; they must be replaced", says Pflugbeil, who works at the Academy of Science's Central Institute for Heart and Circulation Research in Berlin-Buch. He says that important positions there have been filled on a "false selection principle", based on ideology rather than achievement, and that the problem is more general.

"But I don't know where to get competent people to replace them", he continues, "or what to do with them when they have been sent home". East Germany already has a labour shortage because of all the refugees who have fled to West Germany, estimated on 26 November at 283,696 this year alone.

Allotting research funds transparently is another top priority. Even research directors do not know how much has been spent in their respective areas across East Germany. Academy and university funds in many fields have not been totalled to give an overview of

SUPERCONDUCTING SUPER COLLIDER

research spending.

The present crisis may tempt the government to cut back on all research, which Jens Reich, a molecular biologist and member of New Forum, believes would be disastrous by prompting East German scientists to sell their services to the highest bidder. West Germany, the most likely outlet, might then come to rely on East German laboratories for routine or labour-intensive measurements. Already it is reported that East German medical patients have been offered to West German pharmaceutical companies for tests of new drugs.

Would East German researchers really sell out in ways like this if support started to dry up? Reich says, "I only know the scientific community here as frustrated and cowardly, so I am not sure how alert they are to this danger". Reich also sees danger in the "robber-baron" mentality that might take over if East German researchers were forced to compete for the little hard currency available.

New Forum is only slowly learning of others' efforts to restructure institutes. Groups of researchers seem to be meeting independently in living rooms across East Germany to discuss restructuring of their institutes. Eventually, New Forum, may coordinate these discussions, but separate groups seem to be thinking along similar lines.

Dietrich Koch, a specialist in artificial intelligence, has led a group planning a new structure for the Central Institute for Cybernetics and Information Processing at which he works. The plan, put forward on 30 October, is already being put into practice in the vacuum formed by the abolition last week of the Academy statute, the legal basis for the organization of institutes within the academy.

Instead, there is now an institute council to give researchers and technical personnel a say in important decisions previously made by the director or handed down from above, including the choice of topics for investigation and allocation of funds between them.

The weak point in the plan, Koch admits, is the future role of the director. If a director should refuse to accept a diminished role or to cooperate with the changes, there could be trouble. Fortunately, Koch says, the director of his own institute has played a leading part in the reforms. "We all thought he was much more calcified than he has turned out to be."

Koch also proposes that the guaranteed funds that all institutes are used to receiving should be eliminated, a change that may meet resistance from researchers. "The old system stifles creativity", he says, since department heads considered new projects proposed by underlings too risky.

Koch and the others even so agree that there must be a basis of socialism in any new structure of East German research. They say that their views enjoy broad support from researchers of all ages. "If we don't do something different from the West, we might as well all go to West Germany."

Steven Dickman

Rumours of trouble denied

Washington

ALTHOUGH the House of Representatives and the Senate finally agreed this year to authorize \$225 million to start building the Superconducting Super Collider (SSC), the 20 TeV, 53-mile circumference proton-proton accelerator, the high-energy physics community and interested politicians still evidently feel that the project's future is somewhat precarious. This nervousness last week led Representative Joe Barton (Republican-Texas), whose district includes Waxahachie, the proposed site of the SSC, to pay a visit to the Department of Energy (DoE) and hold a press briefing with the aim of calming fears of cost over-runs and technical difficulties hinted at in an otherwise insubstantial story in the *Washington Post*.

The gist of the newspaper story was that SSC director Roy Schwitters had asked the DoE for as much as \$2,000 million over the estimated cost of \$5,900 million to modify and augment the SSC in order to overcome technical troubles with the prototype superconducting magnets. There was also a suggestion that SSC physicists wanted to redesign the injector for the main accelerator ring: in the conceptual SSC design, now four years old, the injector was to feed protons at 1 TeV into the main ring, which would boost them to 20 TeV; now, according to the *Washington Post*, the plan was to raise the injection energy to 2 TeV, easing the task of the main ring magnets.

But Barton, after talking to Schwitters and Deputy Energy Secretary W. Henson Moore, said flatly that there had been no request for extra money, and that a change in the injection energy was merely one of a number of ideas that were being considered in adapting the conceptual design to a final design specific to the Waxahachie site. Barton added that there was also a possibility of reducing the

LASER TECHNOLOGY

Tamper-proof passport on the way

Sydney

AUSTRALIA is about to begin marketing a counterfeit-proof passport. The Department of Foreign Affairs and Trade has developed a laser-printing technique and a special plastic laminate to make what they claim is the world's finest tamper-proof document.

In a conventional laminated passport, the photograph is attached to a page and sealed on by a plastic screen, but the screen can be removed and a new photograph inserted. In the new technique, which works only in black and white, photographic images and other details are printed by laser onto an adhesive reversed-

length of the main ring magnets from 17 metres to 12, but that this was being considered, among other reasons, because shorter magnets would be easier to install.

Little of this is in fact new. Even when the conceptual design was released, there was talk of changing the injector energy to 2 TeV. SSC spokesman Russ Wylie said that this option had received extra support from computer simulations which indicated that a higher injection energy would improve beam quality. And he added that although the first prototypes of the main ring magnets did not produce a magnetic field of sufficiently high uniformity, later models were doing well. He emphasized that the magnet design is still changeable, and will be made final only in a year or two, after collaborative work with the companies that will mass-produce the thousands of magnets needed.

The \$225 million authorized for this financial year is being partially held back by the DoE until it has approved site-specific SSC design, which Schwitters hopes will be ready by the end of the year. Both Wylie and a DoE spokesman agreed that the advertized cost ceiling of \$5,900 million was a solid one, and that changes in the injector, for example, are unlikely to win DoE approval, no matter how good the technical arguments, if they increase the cost.

Although, according to Barton, the *Post* story set off no political fires, he and others were evidently anxious to stamp out the smouldering rumours before they had a chance to flare up.

But the incident did bring out from several quarters official declarations that the SSC will come in at or below budget, statements which may haunt Schwitters and others should the design changes now being pondered belie more serious problems than have been publicly admitted.

David Lindley

image film, which is then ingrained into the glue of the passport laminate.

The Australian government, according to director of passports Ted Radclyffe, has taken out a provisional patent on the process, and a licensing agreement has been signed with the 3M company and Gestetner, developers of the laser printer. Radclyffe says that the United States, New Zealand and Singapore are likely to be early investors and that with expanding markets worldwide for other security printing applications, such as drivers' licenses and identity cards, the technique is "likely to make Australia a lot of money".

Tania Ewing

UK blood screening begins

London

LARGE-scale testing of UK blood samples for the presence of human immunodeficiency virus (HIV) will begin in January. The samples will have been taken for reasons other than HIV testing and will, says the Department of Health, be "anonymised", so that it will be impossible to trace a positive result back to an individual. But, because information about the testing will be made available, there is a danger that those most at risk will be under-represented.

There will be a number of different studies. Of the first two to be set in motion, one will survey blood taken during pregnancy at antenatal clinics and the other will use samples from sexually transmitted disease clinics. Later in 1990, tests will start on blood from certain categories of patients at some general hospitals and on blood of newborn infants, in the form of the dried spots of blood on Guthrie cards used for phenylketonuria screening.

The basis for the programme of anonymous testing is laid out in the 25 November *British Medical Journal* by O. N. Gill of the Public Health Laboratory Service AIDS Centre and two colleagues. If specific consent has to be sought, as when the donors of samples are identified, high-risk individuals are much more likely to decline than those at low risk, they say. Anonymous testing can avoid that bias as it does not necessarily require consent.

Nonetheless, the Department of Health has decided that patients will be told about the surveys by means of leaflets and posters. Precisely how that is put into effect has not been decided and may be left to local authorities. The extent to which this will re-introduce the bias associated with systems of consent is a matter of concern to some observers.

To maintain the anonymity of the blood samples, only limited information will be attached to them. At a minimum, this will be the sex, age and broad geographical location of the person from whom the sample was drawn. Samples from sexually transmitted disease clinics should also carry with them information on the donor's sexual orientation, broad clinical diagnosis and intravenous drug use, say the authors of the BMJ article, but whether that will be available without consent has yet to be determined. "The surveys are an important step forward" says Professor Roy Anderson of Imperial College, a leading advocate of anonymous testing, "but only a beginning". The best source of information would be cohort studies without consent in sexually transmitted disease clinics, he said.

The programme of testing announced last week follows a decision in favour of anonymous tests made just over a year ago by the UK government, despite some residual opposition. The Medical Research Council was charged with designing the surveys. Their results will be coordinated by the Public Health Laboratory Service AIDS Centre and the Department of Genito-urinary Medicine of University College and Middlesex School of Medicine. Several hundreds of thousands of samples are to be tested over the next few years.

Peter Newmark

■ A concerted campaign to persuade the UK government to compensate the 1,200 UK haemophiliacs who became infected with HIV through contaminated blood products has achieved a measure of success. While denying liability, the UK government last week announced a donation of £19 million to the Macfarlane Trust which will administer the handout.

Two years ago the government gave £10 million to the same trust. □

DAHLEM CONFERENCES

Last-minute reprieve

Munich

ENDANGERED in June by the withdrawal of a long-time sponsor, the world-renowned Dahlem Conferences have been rescued by the Free University of (West) Berlin. There will be no interruption of the conferences, which have been held four times a year for the past 15 years.

When the previous sponsor, the 'Donors Association for Promoting Arts and Sciences in West Germany' (*Stifterverband für die Deutsche Wissenschaft* or SV) withdrew its support earlier this year, an alarm went out in the international research community that may have helped to save the conferences (see *Nature* 340, 86; 1989). More than 150 letters poured in

to the governing mayor and the Senate of West Berlin in support of the conferences.

The new arrangement with the Free University will ensure the conferences' future for at least four years. The arrangement meets all the conditions set by the conferences' administrator Silke Bernhard, who said she was overjoyed with the solution. The agreement provides for an independent budget for the conferences, supplied by the West Berlin Senate and various donors. Even in times of need, the university will not be able to cut the budget. In addition, the jobs of the conferences' several employees will be guaranteed under all circumstances.

Steven Dickman

BRITISH RESEARCH

Cheery tone at SERC

London

THE British Science and Engineering Research Council (SERC), the largest of the five research councils supporting work at British universities, seems deliberately to be seeking to lift the gloom over academic research in an up-beat review of activities up to the end of March this year.

Professor E. J. W. Mitchell, SERC's chairman, welcomes the British government's decision that spending this year and in the two following years should be increased by 1 per cent above previous estimates, saying that the result will be to relieve shortages that would otherwise have become plain in the provision of research grants, equipment and subscriptions to international agencies.

But Mitchell also says that the increasing cost of research must be acknowledged if the "seeds sown in the 1980 settlement are to germinate".

Mitchell's introduction includes an explicit commendation of the work of SERC's establishments, most of them founded to provide research facilities for general use by universities. The annual report notes that no country is able to pursue a rounded programme of research exclusively through universities and other academic institutions, and notes that by SERC's policy of allowing that 10 per cent of the cost of in-house research should be spent on basic research, the establishments contribute the equivalent of 160 fulltime researchers to the British research enterprise.

This statement seems intended to counter criticisms that SERC spends too great a proportion of its resources on in-house activities.

Of total expenditure in 1988-89 of £365.5 million (up by £8 million compared with the previous year), SERC spent £123.6 million on research grants (an increase of £2 million) and £123 million on providing grants for graduate students (an increase of £1.5 million). Subscriptions to international organizations such as CERN increased by £3.5 million to £90.4 million over the year, while spending at the council's own establishments and head office increased by £3 million to £123.6 million.

Over the year, spending on engineering research seems to have declined significantly (by more than 3 per cent to £11 million), but there seems to have been a healthy increase in the funds spent by the Science Board, which supports general research, from £95.2 million to £104.7 million, reflecting the general opinion that mainstream science has too often been short-changed in the pursuit of specialized programmes. □

HEPATITIS VIRUSES

Blood donors to be screened

Tokyo

THIS week, Japan's Red Cross is expected to start screening all blood donors in Japan for hepatitis non-A non-B viruses using an antibody test kit developed by the US company Chiron. This will be the first nationwide programme in the world for screening this type of hepatitis virus and will give Chiron a commanding lead over other companies in Japan, where hepatitis non-A non-B is a major cause of liver disease and cancer.

Between two and four million people in Japan are believed to be carriers of hepatitis non-A non-B viruses. Every year, about 200,000 people, 10–15 per cent of blood transfusion recipients, are infected with these viruses. They cause 60 per cent of Japanese cases of hepatitis (about 720,000 people) and 40 per cent of cases of cirrhosis of the liver (100,000) and liver cancer (7,000). The viruses cause 16,000 deaths a year in Japan, according to statistics for 1988 from the Ministry of Health and Welfare. But the viral agents responsible, of which there are several, both blood-borne and orally transmitted, have not yet been fully identified.

Last year, Chiron partially isolated and characterized a blood-borne viral agent that is thought to be one of the major causes of hepatitis non-A non-B. And last January the company provided the Japanese Red Cross with 10,000 samples of an ELISA (enzyme-linked immunosorbent assay) test kit developed in collaboration with Ortho Diagnostic Systems of the United States for trial screening.

According to results reported at an international symposium in Tokyo in September, the test kit detected antibodies in about 75 per cent of post-transfusion acute non-A non-B hepatitis patients. And the Red Cross has decided to begin immediate nationwide screening using 800,000 test kits imported through Ortho Diagnostics Systems' subsidiary in Japan.

The kits are being provided free of charge because the US subsidiary has yet to receive marketing approval from the Ministry of Health and Welfare. But approval is expected shortly and the free samples are a good marketing strategy, according to Mitsuru Miyata, editor of the newsletter *Nikkei Biotechnology*.

Two Japanese research groups backed by industry are in hot pursuit of Chiron. A group led by Professor Terukatsu Arima of Kagoshima University (formerly of Okayama University) which has links with Tonen (formerly called Toa Nenryo Kogyo), an oil refiner, has isolated clones that react positively with 60–70 per cent of serum samples of patients of transfusion-associated chronic hepatitis non-A non-B. And Toshio Shikata of Nihon University, who is collaborating with the Chemo-Sero

Therapeutic Research Institute (Kaketsuken) in Kyushu, has also isolated one clone.

Both research groups have applied for patents for test kits and emphasize the difference between their results and Chiron's. But the limited data released so far suggest more similarity than difference with the US company's results, according to one expert observer of the competition.

The market for blood-screening kits in Japan is potentially enormous because Japan has about 8 million blood donors a year. But the Red Cross, which monopolizes screening, traditionally gets test kits at considerable discount and sales are expected to amount to only several million dollars as with AIDS screening kits.

At the same time, Japan's blood-collection centres are not so familiar with ELISA antibody test kits of the type developed by Chiron and Ortho Diagnostic Systems. So Ortho Diagnostic Systems, in collaboration with Fuji Rebio, is developing a test kit based on Fuji Rebio's aggregation method, which is used for screening AIDS and adult T-cell leukaemia (ATL) viruses in blood donors in Japan. If all goes well, the kit could be on sale in Japan in the second half of 1990.

The market for kits to diagnose patients is slightly larger than that for blood screening and is expected to amount to about ¥1,000 million (\$7 million) a year, as in the case of hepatitis-B virus. But, in tests in hospitals around Japan, Chiron's ELISA kit showed positive results with only about 35 per cent of patients with sporadic non-A non-B hepatitis. The comparatively low percentage of positive results compared with post-transfusion patients is a mystery and has given Chiron's competitors hope that they can develop alternative tests.

But by far the biggest market lies in the development of a vaccine. Chiron has set up a joint venture with Ciba Geigy, called Biocine, for this purpose, and is keeping a tight lid on all information concerning the approximately 30 per cent of its cloned DNA (the part coding for structural proteins) that can be used for vaccine development.

In Japan alone, a vaccine for hepatitis non-A non-B is expected to have annual sales of £4,000–5,000 million (\$30–35 million), comparable to sales of the vaccine for hepatitis B which at present leads the vaccine market in Japan. There are also huge potential markets for a vaccine in other parts of Asia. Development of a vaccine is probably still several years away, according to one expert observer, but Chiron is getting closer to being the 'ultimate winner'. **David Swinbanks**

CHINESE REPRESSION

Students in US may be able to stay longer

Washington

THE US Congress last week passed a bill that would allow Chinese students and scholars staying in the United States under temporary J-1 visas to remain here after their visas expire. But the administration opposes the bill and the president may veto it before the deadline of 2 December.

The bill, sponsored by representative Nancy Pelosi (Democrat-California), is one of many introduced in July shortly after the Chinese People's Liberation Army shot unarmed protesters in Tiananmen Square but is the only one so far which is making any progress through Congress. It would waive the two-year residency requirement on J-1 visas, held by most students and visiting scholars, which stipulates that the holder must return to the country of origin for two years before applying for another visa. The new rule would allow the holder to remain in the United States and apply for an extended temporary stay or permanent residence.

But in a statement released last week, the Department of State said it was strongly opposed to the bill on the grounds that it could damage academic exchanges between the two countries, which had been increasing until the government crackdown in June. If it becomes law it is "extremely unlikely" that the Fulbright exchange programme, which has been suspended since June, will be resumed and the impact on non-governmental exchange programmes might also be very severe, it said. The statement said that although the administration sympathizes with the reluctance of Chinese students to return now, it is also concerned that their permanent loss to China might prevent future visits by others.

The bill has strong support in Congress, however. It was passed unanimously in the House of Representatives and with only a few dissenters in the Senate. A spokesman for Pelosi says that there is probably enough support to ensure that if President Bush vetoes the bill the veto will be overridden by a two-thirds majority in Congress.

Christine McGourty

TECHNOLOGY TRANSFER

Science park for Sri Lanka

Bangalore

THE Arthur Clarke Centre for Modern Technologies will soon develop a 'technology park' designed to give industry in Sri Lanka better access to international technological expertise. The park will be located near the Katubedda University, Moratwa. Different groups in the park will be devoted to the design of new products, the analysis of industrial processes, and software development. **Radhakrishna Rao**

Government must spend

London

MORE manpower is urgently needed for research into the causes and effects of global warming, according to a new report from the Select Committee on Science and Technology of the House of Lords, the Upper House of the British parliament*.

At the United Nations' General Assembly on 8 November, Prime Minister Margaret Thatcher pre-empted one of their Lordships' recommendations to some extent, by announcing the establishment of a National Centre for Climate Modelling at the UK Meteorological Office in Bracknell, Berkshire. A spokesman for the Department of the Environment said that the centre would cost £5.5 million in its first year.

But some of the observations in the report could be construed as critical of the government's contribution to research funding, already projected to fall by £250 million over the next three years—a drop of 30 per cent in real terms (see *Nature* 342, 3; 1989). The select committee is hopeful that a recent report by the Universities' Grants Committee on the support of meteorology in British universities, and a study by the Coordinating Committee on Marine Science and Technology into the recruitment and training of oceanographers "will help promote the needs of these branches of science". But the select committee views the current shortage of expertise so seriously "that they have decided to conduct an enquiry into scientific and technical manpower in the next session of Parliament".

Several factors could be responsible for the shortage. Research into climate change is still in its infancy, and the numbers involved "have never been large", only about 200 to 300 researchers worldwide. But there is a growing recognition that expertise in fields ranging from mathematics to biology can be brought to bear on climate research, and the sudden demands could outstrip supply.

A key factor could be the failure of universities to attract new talent, combined with a demographic drop in the number of science graduates. Many post-graduate places remain unfilled, and the Committee may find that potential researchers are instead turning towards careers with more tangible rewards: this factor, long recognized, was noted by British Conservative MP and former minister Michael Heseltine, speaking at a meeting of the academic pressure group Save British Science (SBS) last Thursday.

But skill shortages are not confined to Britain, and the committee is looking at ways of fostering endogenous scientific expertise in developing countries, where the rate of increase in carbon dioxide emissions is greatest.

Despite the fact that the global warming observed so far is small and may, in any case, be part of a natural climate cycle, "insurance" policies to limit or counteract anthropogenic effects are needed now, before it is too late to do anything. Current models predict a rise in global mean temperature of between about 1.5 and 4.5 degrees Celsius by 2030, and the Committee notes the many worldwide initiatives to limit the emission of greenhouse gases. Reductions might best be secured through a UN Climate Convention, but each country ought to act unilaterally to reduce greenhouse gas emissions "to behave, in other words, as though some kind of convention were in place".

Scientific issues form the core of the report. In particular, fuller understanding of global warming depends in turn on improved modelling and the acquisition of better data for these models to use. The committee notes the shortcomings of many climate models in current use, and the need to simulate atmosphere-ocean coupling accurately. Research on ocean circulation is thus seen as vital, and is a primary focus of activity at the Meteorological Office. Many of these objectives can be achieved at relatively modest cost, implying that any government funds devoted to climate research would be well spent.

For example, the Science Vote should supply the additional £9.1 million requested by the Natural Environment Research Council (NERC), the balance required to meet the costs to the United Kingdom of £33.5 million for participation in the 7-year World Ocean Circulation Experiment (WOCE). And Britain should participate in the Global Energy and Water Cycles Experiment (GEWEX), a proposal by the World Climate Research Programme (WCRP) to place a lidar on a polar platform satellite in the late 1990s. A space initiative "of particular significance" will be the European Space Agency's ERS-1 oceanographic satellite, due for launch in 1991.

The UK should "make a commitment now" to the proposal for ERS-2, the successor to ERS-1, at a cost of about £50 million over five years.

These funds should not be diverted from existing monitoring facilities: "because the global observing system has been in existence for a long time," the report notes, "there is a tendency to assume that its future is assured. However, there is evidence that it is becoming increasingly fragile and subject, even in relatively prosperous countries, to government economies".

Henry Gee

*Greenhouse Effect. 6th Report from the House of Lords' Select Committee on Science and Technology. (London: HMSO) 69pp. Price £7.20 net.

Dangers for higher education

Paris

IN the draft of a report due to be published at the end of this year*, the education committee of the Organisation for Economic Co-operation and Development (OECD) finds that changes in the ways in which higher education is financed could have unsettling long-term consequences for universities.

Financial stringency, says the report's author, Professor Gareth Williams of the University of London Institute of Education, led most OECD governments to look at ways to reduce public spending on higher education between 1970 and the mid-1980s. In some countries new, non-governmental sources of funding have helped, but only Finland and France expect to see an increase in public expenditure on higher education in the foreseeable future.

The report expresses some concern at potentially unwelcome side-effects of an interest in "market" approaches to funding, where universities adapt the courses they offer to the needs of the private sector, in return for non-government finance. Already increasing in the United States and Britain, under this type of arrangement the public sector remains responsible for non-commercial research, including basic science.

But if, in times of cut-backs, public funds are used entirely to support basic teaching and research, says the report, there is a danger that private sponsors will become the only source of finance for innovation.

A major shift towards this kind of funding would mean increasing numbers of staff on short-term contracts and, consequently, a greater separation between teaching and research. But, says the report, there are, in any case, "growing doubts" about the need for research and teaching to be linked within universities and it anticipates a tendency for large-scale scientific research to be carried out in specialized institutions.

The report also says that it is appropriate for universities to derive an important part of their revenue from student fees, as is the case in Britain. But this is a separate issue to that of subsidies to students. The OECD committee anticipates an increase in the use of loans, especially for courses beyond first degree, but expects that repayment of 20 per cent of fees by graduates "probably sets an upper limit to what is likely to be seen as appropriate in many countries".

The principal problem for higher education in the future, concludes the report, will be the incompatibility between increasing financial selectivity and the fundamental need for diversity in the kinds of higher education institution and courses available.

Peter Coles

* A draft of the report, *Changing Patterns of Finance in Higher Education*, was recently discussed at a meeting of vice-chancellors and legislators in Barcelona, Spain.

IVF: regulation or prohibition?

Anne McLaren

The British parliament will shortly be called upon to decide whether research on human eggs, fertilized *in vitro*, should be permitted or made a criminal offence. Scientists must make clear to their MPs where they stand.

THE first baby to develop from a human egg fertilized *in vitro* was born in 1978. By now the total number of such babies in the world must exceed 20,000, of which at least 3,000 have been produced in Britain. In the ten years leading up to 1978, almost the only research on human *in vitro* fertilization (IVF) was carried out by Patrick Steptoe and Robert Edwards: it was their experiments, using eggs donated for research by women volunteers, that led to the birth of Louise Brown in 1978. Since 1978, many more groups have become involved with IVF research. In Britain, all such work is regulated by the Voluntary (now Interim) Licensing Authority, set up in 1985 along the lines recommended by the Warnock Committee (*Report of the Committee of Inquiry into Human Fertilisation and Embryology*).

Of the 38 IVF centres licensed so far in Britain, 17 undertake some research, and the Authority has approved more than 50 research proposals, all listed in its annual reports. Many of the projects are concerned with increasing the success rate of IVF itself, for example by studying the metabolism and the nutritional requirements of the early human conceptus. This work is also leading to an increased understanding of the causes of infertility and later embryo loss, and is pointing the way to new methods of tackling male infertility due to poor sperm quality.

Single-gene defects

Other IVF research projects are aimed at devising methods of preimplantation diagnosis of single-gene defects responsible for diseases such as cystic fibrosis, thalassaemia, Duchenne muscular dystrophy and Lesch-Nyhan disease. One strategy, using *in vitro* fertilization, would be to remove one or two cells at the 8–16 cell stage, a procedure that appears not to prejudice further development; the DNA from these cells would then be amplified (for example by the polymerase chain reaction) to a level at which standard molecular diagnostic methods could be applied, and only those conceptuses shown to be free of the defect in question would be replaced in the woman's uterus. In this way carrier couples who have a 25 or 50 per cent risk of producing an affected child would no longer have to suffer repeated amniocentesis or chorionic villus sampling, with the agonizing prospect of terminating a wanted pregnancy if the diagnosis is

adverse. The need for late abortions for genetic reasons would disappear, and pressure for germ-line gene therapy to 'cure' or replace defective genes would be eliminated.

The *Human Fertilisation and Embryology Bill* going before parliament seeks to set up a Statutory Licensing Authority, as recommended in the Warnock Report, to oversee IVF and other methods of assisted reproduction. The Authority would have statutory powers, but otherwise would operate along much the same lines as the Interim Licensing Authority does at present. On research, however, MPs will have a free vote as to whether the Authority should have powers to license IVF research projects such as those outlined above, provided that they adhere to strict guide-lines, or whether all such experimentation should become a criminal offence.

Who is likely to support IVF research being made a criminal offence, and why? Some people, even perhaps some MPs, are under a misapprehension as to the nature of the early human conceptus. They envisage IVF research as being carried out on a fetus with arms and legs, a brain and a nervous system, capable of awareness or even suffering. The guidelines recommended by the Warnock Report, adopted by the Voluntary Licensing Authority, and proposed for the Statutory Licensing Authority, in fact lay down that no fertilized human egg should be maintained *in vitro* for more than 14 days after fertilization. This 14-day limit was chosen advisedly. It is early enough to antedate the development of the embryo itself, as opposed to that of the extra-embryonic membranes, so is well before even the beginnings of a heart, nervous system or other organs; it is late enough to allow the type of research that led to the development of IVF as a therapeutic procedure, and that is now required to improve the procedure; and it corresponds to an important landmark in embryological development, namely the appearance of the primitive streak. These first two weeks see the start of development of the extra-embryonic membranes, including the placenta, that are going to support and nourish the future embryo. At the end of this pre-embryonic period, 99 per cent or more of the conceptus (the total tissue derived from the fertilized egg) is made up of these membranes, and only a small group of uncommitted cells remains to

form the embryo that will develop into the fetus and finally the baby. Sometimes no embryo forms and the pregnancy fails before even the menstrual period is delayed; occasionally two primitive streaks form, giving rise to twin embryos.

Later stages

The report of the Polkinghorne Committee (*Review of the Guidance on the Research Use of Fetuses and Fetal Material*), published earlier this year, makes a number of recommendations about research on fetuses and fetal material from later stages of gestation. Research on intact living aborted fetuses would be prohibited; research on dead fetuses would be allowed under strict guidelines. Death is defined by the absence of spontaneous respiration or heartbeat; fetal cells would of course continue to live for a period after the death of the fetus. The regulations would not apply to the placenta or other extra-embryonic membranes. The Polkinghorne recommendations appear to be consonant with the Warnock recommendations for the pre-14-day conceptus, with the important difference that they envisage adherence to a code of practice rather than legislation.

The second group likely to vote against licensed IVF research consists of those who themselves hold, or a substantial body of whose constituents hold, the fundamentalist view that the human egg becomes a human person as soon as it is fertilized, and hence cannot ethically be used for research because it cannot give informed consent. Many Catholics are of this opinion. But however much one may respect the views of minorities, in a multi-cultural society like Britain it is really appropriate that such views should constitute the ethical basis of legislation that will have an effect on all sections of the community?

Some fundamentalists would allow research provided the conceptus is to be replaced in the uterus. This is a little like making the first test of a new aircraft-guidance system on a crowded Boeing 747. The Licensing Authority's policy is precisely the opposite: research is permitted only if the conceptus is *not* to be replaced. Experimental procedures should be tested as thoroughly as possible on eggs and pre-embryos donated for research, and only when the Authority is satisfied that the risk of harm to the future embryo is minimal will permission be

given to go ahead with clinical applications.

To be consistent (and some are), fundamentalists who wish to ban the use of IVF for research should also ban its use for therapeutic purposes. As the Archbishop of York cogently argued in an earlier House of Lords debate (*Hansard*, col. 1461, 15 January 1988):

I believe that research must continue if *in vitro* fertilization is to continue. One cannot separate them, and I regard as totally unrealistic and indeed immoral any proposal to continue *in vitro* fertilization without a proper backing in research. This is for the simple and basic reason that imperfect techniques without a backing in research are bad practice medically and, I believe, wrong morally.

But any attempt to ban IVF as a treatment for infertility would lead to widespread protests — not least from couples with an infertility problem, who are thought to constitute at least 10 per cent of all couples in Britain today.

The most common motive for opposing IVF research, however, is neither concern for the possible suffering of the conceptus, nor any fundamentalist ethical belief. It is apprehension or fear of what such research might lead to — the 'slippery slope' or 'thin end of the wedge' argument. Some fear that we may have to suffer yet more costly, high-technology medical treatments, together with hip replacements, heart-by-pass operations and kidney transplants. Yet such techniques are here to stay, and only extreme Luddites would wish it otherwise. In fact, the result of IVF research over the past ten years has been to make its therapeutic use not only more successful, but also simpler, cheaper, more cost-effective and available to a wider range of infertile couples.

Others fear the possible science-fiction implications of *in vitro* fertilization. One reads references to 'Svengali researchers', 'Frankensteins' and 'the Sorcerer's Apprentice'. Improving the success rate of IVF is all very well, people say, as perhaps is preimplantation diagnosis to avoid having to abort defective fetuses — but will this not lead to genetic manipulation of human eggs, or to the production of identical individuals by cloning or to human-animal hybrids? The Interim Licensing Authority has laid down that research proposals along any of these lines would under no circumstances be considered acceptable, and no IVF centre has shown the slightest interest in pursuing such research. Scientists in Britain have long been accustomed to licensed research in the field of animal experimentation, and infringements of Home Office regulations are rare. Without approval from the Interim Licensing Authority, no IVF research project would get official funding; nor, one hopes, would its results be published by any reputable journal.

Slippery slopes are not unique to

The question of regulation versus prohibition raises wider issues than just those of IVF research, wider even than the welfare of infertile couples and carriers of crippling genetic defects. Scientists should make their voices heard.

science. They are a general characteristic of our society, and we have well-tryed and long-established methods of making sure that we do not slide too far down them. Regulations, licensing authorities, inspectors, surveillance — the trappings of slippery-slope control may be irksome, but they are remarkably effective. It rarely becomes necessary to prohibit people from using the reasonable and useful sections of the slope, for fear of what lies below. Slippery-slope arguments are good arguments for regulation and surveillance, but bad arguments for total prohibition.

The history of anatomical dissection of human corpses, although by no means a close parallel to research on the early human conceptus, makes an interesting study in its own right. From the time of Henry VIII onwards, English law prohibited the dissection of any bodies other than those of executed criminals. The science of anatomy flourished in Europe, but remained backward in England. In the early 1800s, there were a thousand medical students in London and another thousand in Edinburgh, yet on average fewer than 80 criminals were executed each year. Not surprisingly, grave-robbing and body-snatching became lucrative trades. By the mid-1820s, 200 people in London alone were engaged in the illegal procurement of bodies for medical schools, supplying them with around 800 bodies a year. Armed guards began to be placed on the graves of the wealthy, the price of a body rose from £2 to £14, and medical students increasingly had to go to Paris for their practical work in anatomy.

Then the resurrectionists ('Sack-em-up-Men') began manufacturing their own corpses. The famous Burke and Hare murder took place in Edinburgh in 1828. Parliament responded by setting up a Select Committee to enquire into the best means of providing a legitimate supply of bodies for dissection. In 1829, the Anatomy Bill was introduced to Parliament. It was passed in the House of Commons, but in the House of Lords it was opposed by a group of peers headed by the Archbishop of Canterbury, and was withdrawn. In 1831, another murder came to light, this time in London. A 14-year-old Italian boy who made his living by showing white mice was strangled, and his body offered to King's College. Public opinion was aroused, and the parliament-

tary response was immediate. In 1832 the first Anatomy Act was passed. It regulated the supply of bodies to universities, with strict legal safeguards and government inspectors. Licences were issued, and no licensed person could be prosecuted for having a body in his possession for anatomical purposes. The murders ceased, the resurrectionists went out of business, students were soon provided with excellent facilities for anatomical study, and Britain began to lead the world in surgery as new life-saving operations were pioneered.

What might be the consequences of prohibiting IVF research? It is most unlikely that research would be pursued illegally, or that any illicit trade in human fertilized eggs would spring up. 'Egg snatching' would hardly be lucrative. Most research workers in Britain would change to other areas of science, though a few would doubtless pursue their studies in other countries, perhaps in France or Italy. Clinical applications of IVF would be severely hampered, and improvements in the success rate would be slower in coming. With preimplantation diagnosis, there would be a risk that new procedures would be tried out clinically (this would not be a criminal offence under the Act) before they had been tested *in vitro* to establish their safety and reliability.

Britain is respected throughout the world for the sensible regulatory approach that it has adopted in this sensitive area, and for the innovative and productive research that has resulted. The research is all licensed and open to scrutiny, and is at present unrivalled in any other country. Whether this situation will continue will depend on how our parliamentary representatives vote.

To appreciate the pros and cons of this issue, no specialized knowledge of embryology or moral philosophy is required. If it were, parliament would hardly be the best body to take the decision. In a democratic society, there is no reason why the views of scientists on ethical matters should carry more weight than the views of anyone else; but nor should they carry any less weight. Yet MPs' mail bags — said to be the major determining factor in the parliamentary process — carry relatively few letters from scientists. Writing scientific papers is distraction enough from one's research; writing letters to MPs comes a long way down the list of priorities.

The question of regulation versus prohibition raises wider issues than just those of IVF research, wider even than the welfare of infertile couples and carriers of crippling genetic defects. Scientists should make their voices heard. □

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Alchemy of matter and of mind

Richard L. Gregory

It seems strange that Isaac Newton, for all his scientific achievements, was so attracted to the occult. Yet his vision of the power of symbols has resonance today.

WE may now see alchemy as 2,000 years of scientific failure. Yet the strange fact is that Isaac Newton, the greatest of all scientists, laboured for more than 30 years on alchemy. He built furnaces for his endeavours at Trinity College, Cambridge, collected a huge private library, copied manuscripts in his own hand and compiled dictionaries of myth and occult symbols, using all the power of his unique genius to understand matter and matter's relation with mind.

Newton wrote a million words on alchemy, yet did not publish a single one. Why should this be? One interpretation is that he doubted that his experiments really worked. Another, possibly more likely interpretation, is that he regarded the potential powers of alchemy as too dangerous for public knowledge. This is the view taken of Newton's silence by B. J. T. Dobbs in 1975 (ref. 1). Golinski² points out that secrecy was a common characteristic of alchemists, who disliked sharing 'noble' knowledge with the 'base' public. Something of this élitism persists in science today.

It seems bizarre that Newton — in his prime an alchemist — became, as Master of the Royal Mint, keeper of the nation's gold. How could this great scientist believe that prolonged gentle heat and magic spells could turn sulphur and base metals into gold? Is it possible that he was motivated by promise of gold, as a rival to the pursuit of truth? This is a shocking, even blasphemous thought. But is it correct? Although Newton's mother was fairly rich (his father died before his birth), she refused to pay for his full undergraduate status at Trinity, causing the young Newton to suffer from literally degrading poverty. As Lucasian Professor of Mathematics, he received an annual income of £160. Following his incredible scientific achievements at Cambridge, he became Warden and then Master of the Royal Mint, where he was paid according to the number of gold coins the Mint produced, receiving as much as £3,500 per annum. Although still entitled to it, he did not for a time trouble to claim his Cambridge salary, which must by then have seemed insignificant.

Was the mainspring of Newton's life attaining the wealth of gold, the greatest work of science, the *Principia*, being but a bauble on the way to riches and fame? What makes this hypothesis absurd is surely Newton's immense success as a

scientist. If the *Principia*, the *Opticks* and the rest of his scientific achievements had been unremarkable, the hypothesis would surely be perfectly reasonable. History should be thought of as setting up hypotheses, but clearly hypotheses of history are hard to test. Often plausibility can depend on psychological generalizations, which are particularly hard to apply in the case of a creative genius.

Newton was not alone among great seventeenth-century scientists in believing that transmutations of 'base' metals into 'noble' gold should be possible. All substances were then supposed to be made of the four aristotelian elements — which might better be called 'principles' (air, earth, fire and water) — imposed on the central *prima materia*. As it was incorruptible, gold was considered to have special status; nevertheless it seemed to be difficult, rather than impossible, to create or transmute it. It was known well before the seventeenth century that gold could be dissolved by ordinary *aqua regia* (a mixture of nitric and hydrochloric acids), but it was thought this did not change the nature of the gold, merely dissolving it essentially unchanged into small particles.

The possibility of transmutation of the noble metal was accepted by such distinguished seventeenth-century scientists as Robert Boyle and G.W. Leibniz as well as Newton. The 'father of chemistry', Boyle, destroyed alchemy's conceptual

basis by denying Aristotle's four elements (or principles) in favour of defining 'elements' as substances that by all chemical means cannot be changed to other substances. Yet as late as 1782 James Price, who was elected a fellow of the Royal Society in 1781, with some mysterious powders turned sulphur into 50 or 60 times as much silver or gold in a public demonstration which passed contemporary assay tests. Concerned with the honour of the Royal Society, its president, Sir Joseph Banks, requested Price to repeat the experiment before the fellows. But this time it failed to work, and Price took poison, committing suicide and dying at the demonstration.

The possibility of transmutation of elements alchemically did not die entirely, even after the advent of John Dalton's atomic theory of chemical elements in the nineteenth century. Paradoxically, the discovery in the early twentieth century that elements can transmute by radioactivity finally destroyed alchemy. This seems a rare case of a successful prediction serving to destroy the hypotheses supporting it. Strictly speaking, the transmutation of atoms by radioactivity makes nonsense of the chemical meaning of 'atom' and 'element'. These definitions should have been changed to allow transmutation, as a logical possibility, after the event. This was avoided by denying that effects of radioactivity are 'chemical'. The old alchemists had no such semantic

IMAGE
UNAVAILABLE
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REASONS

Alchemical symbol of transmutation of base metal (Earth, at bottom) into Gold (the Sun) and Silver (the Moon), through the agency of the dragon (Mercury, or volatility). The illustration appeared in *Theatrum Chemicum Britannicum*, by Elias Ashmole; Newton is known to have had a copy of the book in his library.

or logical problem with their aristotelian sense of 'elements' as mixtures.

Newton's concern for the dangers of alchemy may have prevented him from publishing his findings in what can be seen as a dramatic seventeenth-century parallel to the débâcle over present-day claims that nuclear fusion can take place in a test-tube at room temperature to generate heat beyond that which can occur by chemical means^{3,4}. Boyle claimed that philosophical sulphur could heat gold by alchemical power in 1675, the year Newton first met him. Newton was so disturbed by Boyle's claim that he wrote privately to the Secretary of the Royal Society, Henry Oldenburg, urging that such alchemical secrets were

not to be communicated without immense damage to the world if there should be any verity in the Hermetic writers, therefore I question not but the great wisdom of the noble Author [the Hon. Robert Boyle] will sway him to high silence till he shall be resolved of what consequence the thing may be.

Newton ends this letter: "I have been so free as to shoot my bolt but pray keep this letter private to yourself".

What exactly was this claim, or discovery, of Boyle's that so worried Newton? It was an alchemical experiment carried out by Boyle which he reported in a paper⁵, *Of the Incalcescence of Quick-silver with Gold*, published in 1675, introduced by Henry Oldenburg:

In this long paper Mr. B.R. [Robert Boyle] enters into an inquiry whether or not the commixtion, or amalgamation, of gold with mercury, is accompanied with a sensible production of heat; which he decides in the negative, in respect to common mercury, and in the affirmative with regard to purified, or (as he terms it) ennobled mercury. I took, says he, to one part of the mercury, sometimes half and sometimes an equal weight of refined gold reduced to a calx or subtle powder. This I put in the palm of my left hand, and putting the mercury upon it, stirred it and pressed it a little with the finger of my right hand, by which the two ingredients are easily mingled, and grew not only sensibly but considerably hot, and that so quickly, that the incalcescence sometimes arrived at its height in a minute.

With his usual excellent scientific method, Boyle guards against the possibility of a sensory illusion of heat through irritation of the skin:

I had the curiosity to keep the mixture in a paper, and found not its interposition to hinder me from feeling the incalcescence, though it abated the degree of my sense of it.

He tried it out with successful confirmation both on Oldenburg and on the President of the Royal Society, Viscount Brouncker.

Boyle makes it clear that the mercury he used was not 'ordinary', but he gives no details of it. Indeed, he makes curious excuses: that he lost "a considerable quantity of it"; "the almost sudden death of the only operator I trusted in the

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Isaac Newton — laboured on alchemy.

making of it"; and that he was "diverted by business, sickness, and more pleasing studies". It seems that Boyle shared Newton's fear of this alchemical reaction supposedly producing heated gold not with normal but only with specially prepared 'philosophical' mercury.

Could this seventeenth-century cold fusion-like heat have been due to some impurity? I am no chemist; but presumably modern chemists would prefer an assumption of impurity to one of alchemical effects. Assumptions of possibility are sometimes changed by experiments: cold fusion looks so seductively interesting because it challenges assumptions of impossibility in the 'neo-alchemy' of the splitting of the atom by radioactivity. But, like Boyle's alchemical claim of 300 years ago, it seems that cold-fusion experiments^{3,4} are not sufficiently powerful to shift current assumptions. Some artefact is a more likely explanation than energy for Boyle's alchemy, as well as, it seems, for modern cold fusion.

Following years of work, Newton claimed to have succeeded in developing a 'philosophical mercury' for producing gold, and later an effective 'philosophical sulphur'. We find a clue to why he saw these developments as possible from his interest in the *star regulus* of antimony, a semi-metal occurring naturally as antimony sulphide, now called stibnite. The crystals are long and thin and generally form into fern-like patterns. But under certain conditions, which the alchemists discovered, they form patterns radiating from a centre, like drawings of stars. It was this star of antimony that fascinated Newton. It was called by alchemists the *regulus* (little king), referring to the star *Regulus* in the constellation of Leo. Newton saw a relation between the 'regulus of antimony' and the star *Regulus*, much as the alchemist Basilus spoke of the star of antimony as having: "a spirit which is its strength, which also invades it invisibly, as the magnetic property per-

vades the magnet". It seems that this greatly appealed to Newton, leading him to think that by analogy with celestial gravity it would draw the 'philosophical mercury' out of other metals. Traditionally, alchemy is closely tied to astrology, as each planet is ascribed a metal, and Newton looked for alchemical relations between substances and certain stars, especially *Regulus*. Wouldn't he have loved to apply the spectroscope to the substances of stars! The characteristic spectral lines of elements and molecules indeed disposed of the long-held 'impossibility' of our knowing what stars are made of.

The alchemists were pioneers in experimental method. But the really interesting question is why experimental science took so long — thousands of years — to become effective. It is possible that progress in experimental science using inductive method and critical experiments was held up by astrology. Controlled experiments were virtually impossible if it was necessary to wait for the planets, the Moon, the Sun and stars to return to the same positions in the sky for each comparison trial of an experiment. So Bacon's experimental method of the *Novum Organum*, published in 1620, which we follow in essentials today, could not be carried out before the demise of astrology. But if this is so, how could astronomy, the ancient queen of sciences, have been so successful? A reasonable answer is that observational astronomy escapes the problem because it does not use controlled experiments. Events happen and patterns form and change in cycles beyond human control, but they can be described and used for prediction. It is these observed cycles that Ptolemy explained mechanistically with his epicycles, and Kepler and Newton made sense of with their mathematical models. On this view, astronomy is a special case, making it the first but atypical science to be successful, despite astrology making experimental method impossible.

Although Newton did not publish explicitly on alchemy, there are strong hints of alchemical ideas in his published work. He was the supreme discoverer of astronomical mechanisms, yet it seems that he looked to alchemy for principles underlying mathematical accounts of mechanisms. Evidently he found the mechanistic account of nature in terms of forces and motions of bodies unsatisfactory as a complete account. It has even been suggested that his interest in alchemy was a revolt against mechanistic philosophy, but this may be too strong. Newton wrote two unpublished papers on underlying principles with rich hints of alchemy in 1699. The first contains the following sentences: "The vital agent diffused through everything in the earth is one and the same". "And it is supremely volatile, which is

dispersed through every place.” “The general method of operation of this agent is the same in all things; that is, it is excited to action by a gently heat, but driven away by a great one, and when it is introduced into a mass of substances its first action is to putrefy and confound into chaos; then it proceeds to generation . . . In a metallic form it is found most abundantly in Magnesia [antimony].”

The second paper is usually called *The Vegetation of Metals*. Westfall⁶ summarizes it (his italics): “His focus in ‘Vegetation’ was the earth and its mineral products. He described how the metallic spirit ascends from the bowels of the earth, is fixed with salts and minerals, when it meets water, and is thereby alienated from its metallic nature. The alienation arises because concretion is not a process of vegetation but only ‘a gross mechanical transposition of its parts’. If the spirits can be freed from their fixed compositions, they can again ‘receive metallic life & by degrees recover their pristine metallic forms’.” Westfall continues that *The Vegetation of Metals* “proclaimed Newton’s conviction that mechanical science had to be completed by a more profound natural philosophy which probed the active principles behind particles in motion”. Newton never abandoned his view that “The whole frame of nature [might] be nothing but aether condensed by a fermental principle”.

Newton’s final account published in the *Queries of Opticks* reads:

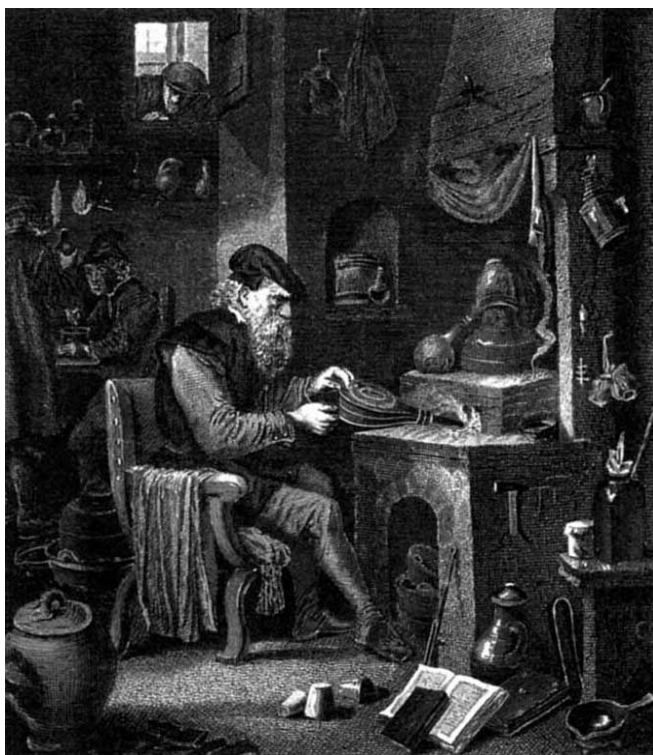
Are not gross Bodies convertible into one another, and may not Bodies receive much of their Activity from the Particles of Light which enter their Composition? For all fix’d Bodies being sufficiently heated emit Light so long as they are sufficiently hot . . .

The changing of Bodies into Light, and Light into Bodies, is very conformable to the Course of Nature, which seems delighted with Transmutations.

Newton was deeply interested not only in matter and forces operating in the material world but also in the mind: how we can sense things and appreciate the principles by which the Universe works. His interest in the mind was bound up with his concepts of space, the aether and absolute motion. He did not simply assume absolute motion but questioned it and sought experimental evidence. Absolute motion in a fixed space, or aether, was important on theological grounds, for he saw space as God’s mind. He thought of the laws of the physical world as God’s ideas, which he might change at any time. Considering that he could discover the laws with mathematics, Newton thought he had a

kind of telepathic communication with God’s mind — which somehow existed in the aether of the space between the stars.

In a very different way, living organisms determine their own behaviour from within themselves. For Newton, the laws of motion of the heavenly bodies lay not in the bodies but in space, because any heavenly body of any composition of the



The alchemist's laboratory, an engraving from the seventeenth century.

same mass would behave the same. The mystery was how the symbols of mathematics could reveal astronomical forces and laws. The hope was that symbols might reveal powers and laws of the mind. Is this so far from modern psychology?

Surely we are close to seeing how the best scientific intellects of the seventeenth century could confuse physical laws of mechanism with the powers of symbols. Yet it does seem odd that Newton thought of the pattern of crystals of antimony as drawing in power and attracting principles of metals, perhaps making gold. There is surely an analogy here with the immense power of symbols in our everyday life. We are just as affected by symbols as by physical forces — by patterns of pictures, words, music and argument. We live (and with modern advertising may drown) in a sea of symbols. As alchemy made no sharp distinction between living and non-living, it is hardly surprising that symbols were seen to have powerful influences in the inorganic world.

For Newton, alchemical notions were an escape from total mechanism and provided hints for bridging matter and mind. Particularly with his work on colour, this problem greatly intrigued him. The mech-

anical philosophy of the problem was set up in the generation before Newton. René Descartes had suggested that mind is essentially separate from matter, seeing the body as a machine controlled by a separate mind, through a mysterious quixotically identified link, the pineal gland of the brain. Newton disliked cartesian dualism of mind and matter. He looked for matter–mind links in the symbols of the experimental theology of alchemy.

The ancient Greeks realized the power of symbols and that symbolic processes can be handled by mechanisms: counting on the fingers (digital), and moving pebbles (calculate) as for the abacus. Yet this ancient notion of matter mechanisms carrying out symbolic processes did not become a paradigm for physiological psychology until as late as the middle of the twentieth century. Charles Babbage showed in the 1830s that a machine could make its own decisions during calculations and mechanical calculators were well known through the nineteenth century; yet they never seem to be mentioned by psychologists concerned with how the brain works for thinking, learning and seeing. And despite the obvious powers of modern electronic computers for handling symbols, learning and even seeing, explanations in terms of cognitive

concepts are still taboo for many distinguished physiologists. Perhaps they are frightened of being branded alchemists.

Newton saw symbols, in physics and in the mind, as extremely powerful and dangerous. The experiments and symbols of mathematics that produced the atomic bomb show he was right to respect and even keep secret the powers of symbols — even though he could not have anticipated this outcome of alchemy. Perhaps poignantly, on the paradigm of artificial intelligence for putting powers of symbols into machines describing the mind–brain relation, Newton did not refer to the first machine to do mental arithmetic, with meshed metal gears — Pascal’s calculating machine, invented in 1642 — the year Newton opened his eyes to the light. □

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In defence of Shockley

SIR—The obituary of William B. Shockley (*Nature* **341**, 180; 1989) does not do justice to a remarkably creative scientist, focusing as it does on what might be called the aberrations of his later years and almost neglecting his truly creative period between the late 1930s and the early 1950s before he left the Bell Telephone Laboratories. Moreover, it does not say that his intense and (to my mind) ill-conceived concentration on socio-genetic matters occurred after a head-on automobile collision in which he was almost killed. His Palo Alto surgeon, Dr Bert Davis, told me at the time that he was on the operating table for some seven hours in a touch-and-go situation.

I first met Shockley in Palo Alto in 1930 when we were both undergraduates, he at Caltech and I at Stanford. We formed a semiprofessional friendship which lasted until we disagreed on assessments of the actual value of IQ tests.

We both ended up doing graduate work in the eastern United States. Moreover, we both worked in the field of solid-state physics during our most productive research years. He returned to California in 1954 to start his ill-fated semiconductor company which, through the accidents of fate, had a profound catalytic effect on the evolution of Silicon Valley.

Shockley's graduate work at Massachusetts Institute of Technology was carried out under the leadership of John Slater. He joined the Bell Telephone Laboratories in 1936 and there learned to seek novel concepts in the field of electronics — a grooming that paid rich dividends. Early in the Second World War, both he and James Fisk, later director of the laboratories, carried out a preliminary investigation of the design of self-sustaining nuclear reactors — an endeavour that was inevitably superseded by the work of others once the Manhattan Project was formed. He spent the remainder of the war working in operations research and became a key adviser to General H. H. Arnold, head of the Air Corps. His imaginative analysis of the effects of the conventional bombings of Japan proved remarkably accurate when on-site observations were made later.

From 1945 until he left the laboratories in 1954 were his years of greatest scientific creativity, where he displayed remarkable gifts for interrelating theory and phenomenology in bench-top observations — gifts which to me at least were reminiscent of those of Enrico Fermi. During this period, Shockley carried out informative investigations in connection with magnetic domains, the factors involved in photolysis of the silver halides and the properties of dislocations. The climatic work, of course, was that carried out in

cooperation with John Bardeen and Walter Brattain, whose experience was essential to its success. Shockley's first solo attempt to develop the equivalent of what is now termed the field-effect transistor failed for reasons that Bardeen was in an ideal position to appreciate from his research and experience on surface states of solids.

One can perhaps find in his earlier outlook towards social matters the roots of the attitudes that led him into his justly unpopular and clearly unprofitable excursions into what he regarded as the exact science of racial genetics. Shockley always kept his own counsel and never sought social companionship for its own sake. I am inclined to believe, however, that the residual effects of his near-fatal accident cannot be ignored in evaluating his activity in later years.

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How courageous?

SIR—One must applaud the fact that he shows some concern about the injustices of the apartheid system, but when your correspondent John Ormerod "sticks out his neck" (*Nature* **341**, 99; 1989) this is a somewhat different affair than when South African academics (whom he does not wish to support) do so — *vide* the case of David Webster, murdered for his stand against racism. Further, Ormerod's chosen method of opposition ("saying No to science from South Africa") avoids entirely the issue of who if anybody should teach black South Africans at the tertiary level — does he believe tertiary education is a luxury they do not need? Or is it just that he thinks they do not need to know any science?

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SIR—I have just read the letter by Jan Ormerod of Oslo (*Nature* **341**, 99; 1989) explaining how courageous he is to say "no" to science from South Africa. In the special environment in certain university circles in Norway, I think it would take more courage to say "yes". These people seem to base their opinions only on information from the 'victims' and activists living in what are called the frontline states. As proof of Norway's stand (or lack of it), I have the following story to tell.

This summer, the Agricultural University at Aas held a symposium with participants from all over the world. The subject

was "Preharvest sprouting in cereals". A representative from South Africa was denied participation. When I asked why, I was told that Norwegian boycott laws disallow South African participation in scientific meetings. But, I was told, "this does not apply to medicine, in which field South Africa has so much to offer".

In other words, we boycott only if it does not hurt us. It has been much publicized that South Africa has its flaws, but people there are certainly working to change things for the better. Remember, Rome was not built in a day. South Africa has a lot to offer, not only in medicine but also in agriculture, which in the near future will have to cope with the problems of feeding the world's ever-increasing population (85 million a year) on a steadily decreasing acreage. I hope Ormerod and the Norwegian policymakers will soon realize they must change their attitude towards South Africa — that would really help the 'victims' too. Closing doors seldom solves problems, it just creates them.

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Hidden dangers

SIR—It is easy to write, as does Bo Wahlström (*Nature* **341**, 276; 1989), of banning carcinogenic, mutagenic and teratogenic chemicals. Few, however, realize how widespread these properties are (at least in animal experiments).

Consulting a standard work (Sax, *Dangerous Properties of Industrial Materials*), I discover that the following are suspect carcinogens and both mutagens and teratogens: ethanol, fructose, lactose and maltose. If I restrict myself to experimental teratogens and mutagens, sucrose, sodium chloride and retinol (vitamin A) are added to the list.

All these must, then, be placed well up Wahlström's multiproblem list for imminent banning.

Does Wahlström propose to ban them? If so, how? If not, why not, by his criteria? If he will restrict his choice to problems revealed by human epidemiological studies, he will still have to ban retinol, and its precursor carotenes — which prove ever so slightly fatal for Swedes.

The probability is that, given suitable experimental techniques and appropriate test species, one can show that any and all substances are carcinogenic, mutagenic and teratogenic. Unless, of course, they are more immediately deadly by other means. Perhaps it would be more appropriate to ban chemicals that show none of the above properties, and leave ourselves something to eat.

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Missed opportunity in biology

The US National Academy of Science's study of opportunities in biology research is a thrilling tale and a lost opportunity. It says too little about research policy, and nothing about regulatory impediments to research.

Why does the US National Academy of Sciences repeatedly attempt to rewrite the late Vannevar Bush's great work *The Endless Frontier*? That is the question provoked by the appearance this week in Washington of the latest in the academy's monumental studies of research opportunities in a selected field of science, this time biology. The question is simply answered. *The Endless Frontier* was one of the decisive influences in persuading the US Congress that basic research should be supported generously, so is it not prudent to retell the tale lest the Congress should forget?

That seems to be the calculation, but the arithmetic is wearing thin. The Committee on Research Opportunities in Biology consists of 20 people. It was helped by 11 panels on special topics with at least half a dozen people each, by a hundred miscellaneous contributors and reviewers and a staff of six. It has spent five years compiling an account of the latest phase of the reductionist revolution that falls between three possible goals: a piece of popular science writing, a sales pitch at prospective graduate students and an attempt to secure the exemption of basic research in biology from the constraints of the Gramm-Rudman Deficit Reduction Act.

Peter H. Raven, director of the Missouri Botanical Garden and chairman of the committee, says that the book (which runs to 424 pages) has been written for "biologists; policymakers both (*sic*) in government, universities and in industry; and other scientists . . . who may interact with biologists", which is fair enough. The text is indeed well suited for setting a little knowledge, or a patchy knowledge, in a broader framework. But the absence of a list of references will frustrate many serious readers, especially because the tone of restrained excitement has been heightened by vignettes of discovery, in figure-legends and boxes, told in baby-talk often so arch that they will be mystifying without further reading.

The tale is, of course, every bit as exciting as the committee repeatedly proclaims. It is true that "all fields of biology are being revitalized" by the coincidence of the flowering of molecular biology with the arrival of other new techniques, from data-processing to NMR. It is true that biotechnology will "provide the basis for the accumulation of wealth at many levels" and may also yield means of treating conditions as different as AIDS and

some forms of cancer. Evidently a better understanding of the human nervous system will throw light on the causes of psychiatric diseases, and possibly on their treatment. Gene transplantation will yet transform animal and crop husbandry. And so on, and so on.

Nor has the excitement blunted the committee's judgement. Far from suggesting that all problems have now been solved, the committee is at pains to describe the questions that remain and the difficulties to be overcome. Protein folding, the initiation of cell division and the roles of learning and/or memory in animal behaviour remain basic puzzles. Similarly, understanding self-incompatibility in plants is an obstacle to the more deliberate application to agriculture, while the mechanism of diseases as apparently mundane as osteoarthritis must be determined before more deliberate therapies can be developed. But the outlook is encouraging.

The Raven committee might well have made more of the pace of technical innovation in molecular biology. It is too easily forgotten that even gel electrophoresis was still an awkward technique (chiefly for the analysis of proteins) a quarter of a century ago. Since then, discoveries in molecular biology important in themselves (restriction enzymes and reverse transcriptase, for example) have spawned their own technical revolutions (for determining the sequence of nucleotides in a nucleic acid molecule or for copying RNA into DNA respectively). Now it seems that there is a radical innovation of laboratory technology every few years. There will no doubt be many laboratories not equipped for the latest trick, called PCR, by which a single DNA molecule may be amplified indefinitely, before the next upheaval will be upon them. Is there any other field in which the pace of laboratory innovation has been as rapid?

Most of the other defects of the report are also omissions, but there is an irritating sin of commission — the repetition of declarations such as: "It is vital that the United States provide leadership in this [or that] area". One must suppose that the phrase is aimed primarily at members of the Congress and their staffs, but it is also echoed by a disconcerting argument tucked away in the insubstantial chapter on research policy at the end of the report.

Briefly, the committee notes that, in the international literature of biology in 1973, articles from the United States accounted

for 74 per cent of the 10 per cent of most frequently cited articles, but for only 70 per cent in 1980, while the proportion of frequently cited articles from Japan doubled in the same interval (to 6 per cent). The report muses on the question whether the explanation is more spending by the Japanese government, discovers comfort in the circumstance that in the field of biotechnology patents, "the United States is maintaining its leadership role" (presumably, "lead") and concludes that the United States should strengthen its continuing encouragement of international collaboration "as other countries increasingly emerge as valuable sources of quality research". The conclusion does not follow from the preceding argument, while the chauvinism is out of place.

The remaining policy recommendations are largely inoffensive but also disappointing. There may well be manpower shortages in the late 1990s, predoctoral and postdoctoral training positions are probably too few, it would be good if the numbers of women in biology more accurately represented the sex ratio (and if more academic women had tenure) and so on. There will be general agreement with the plea that something should be done the better to organize the data of biology, both information (sequences, for example) and material (the contents of museums and herbaria) but the Congress will hardly be set alight.

Circumstances change. In Vannevar Bush's time, the general view was that research is, almost by definition, a public good. Now, in genetics (the manipulation of genes and also their mere sequencing), embryology and the use of animals in research, researchers are hampered, rightly or wrongly, by regulation and public fuss. It would have been a public service, especially for the US Congress, if the Raven committee had provided a means by which legislators and their constituents might strike a better (because better understood) balance between legitimate public interests and the regulation of research. But regulation is hardly mentioned.

The committee will no doubt say that its terms of reference mentioned opportunities in, not impediments to, biology research, but that will be a cop-out. These days, the most telling account of the opportunities will not speak for itself, which is why the Raven report reads like an old-fashioned document and is a missed opportunity.

John Maddox

Age of the modern Europeans

Lawrence Guy Straus

Two articles by James Bischoff and four Spanish colleagues in this month's issue of *Journal of Archaeological Science*^{1,2} will again stir up the debate over the origins of modern man. The authors describe stratigraphically coherent series of accelerator radiocarbon dates of well-provenanced samples of charcoal from two modern excavations in northern Spain. There are three chief conclusions.

- That the Aurignacian, the earliest technology generally believed to have been made by anatomically modern, European humans (*Homo sapiens sapiens*), dates to 40,000 years ago, much older than had been previously acknowledged in western Europe.

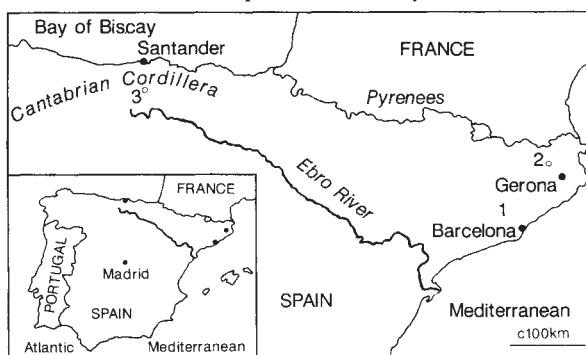
- That the Aurignacian technology may have spread from eastern Europe much faster than usually thought.

- That the transition from the Mousterian to the Aurignacian, at least in Catalonia, was extremely rapid, and consequently the replacement of the Neanderthals by modern humans may have been fast in some regions of western Europe, while in others the two human forms may have existed side-by-side for some time.

Debates about the process and timing of the evolution and geographical dispersal of anatomically modern humans and their associated Upper Palaeolithic technologies have raged particularly fiercely since the formulation of the 'Out of Africa'/'Daughters of Eve' hypothesis, based on measurements of genetic distance and relationships in mitochondrial DNA among modern populations^{3,4}. According to this hypothesis, archaic forms of *Homo*, such as *H. erectus*, were recently (100,000–200,000 years ago) and completely replaced without genetic admixture by modern people who had originated in Africa. On this view, the Neanderthals of early late Pleistocene Europe would be relegated to a dead-end branch of humanity, whereas other palaeo-anthropologists believe that they contributed to the gene pool of modern humans⁵. The 'Out of Africa' hypothesis is supported by palaeontological finds from southern Africa that unfortunately are either equivocally dated (Border Cave) or anatomically equivocally diagnostic (Klasies River Mouth)⁶. The controversy has been further fuelled by the recent thermoluminescence and electron-spin-resonance dating of deposits with anatomically modern human remains at Qafzeh Cave and Skhul in Israel to $\geq 90,000$ years ago — an age about 10,000–30,000 years older than other Neanderthal finds in the Near East^{7,8}.

In Israel, both Neanderthal and *Homo sapiens sapiens* remains are associated with simple stone tools of the Middle Palaeolithic period.

In Europe, the oldest archaeological assemblages with which anatomically modern human remains have been found are assigned to the Upper Palaeolithic Aurignacian 'culture', characterized by specialized endscrapers and burins (gravers) often made on large stone blades, small retouched bladelets and a variety of bone 'points', as well as objects of art and adornment made of stone, bone, ivory, antler and tooth. Artefact assemblages that combine some artefact types and manufacturing techniques of both the Upper and Middle Palaeolithic are called 'Chatelperronian' in France and Spain. Diagnostic human remains are associated with the Chatelperronian in only one site,



Sites of late Mousterian and early Aurignacian technology in northern Spain, now dated to 40,000 years old. 1, Abric Romaní; 2, L'Arbreda; 3, El Castillo.

Saint-Césaire (and, less certainly, in Arcy); they are of a Neanderthal^{9,10}. An analogous transitional cultural tradition in central Europe, the Szeletian, may also have been the work of Neanderthals¹¹. Until now, a consensus reading of the evidence suggested that modern people with an Aurignacian technology had entered southeastern Europe at some time before 43,000 years ago, moved across central Europe in the period between about 44,000 and 37,000 years ago and finally reached western Europe by around 34,000 years ago^{11,12}. After only a brief period of contemporaneity with Neanderthals and their Chatelperronian culture, the modern humans and their Aurignacian culture replaced them by means that remain unclear. The accelerator radiocarbon dates reported in *Journal of Archaeological Science*^{1,2} can be interpreted as calling into question elements of this view of events.

In association with Victoria Cabrera, who is excavating remnant deposits in El Castillo cave (Cantabria, Spain), Bischoff obtained dates of 40.0, 38.5 and 37.7 kyr

BP (before present); these are in correct ascending stratigraphic order within the stratum and all bear a standard deviation range of about $\pm 2,000$ years. The basal Aurignacian layer is separated from the (as yet undated) uppermost Middle Palaeolithic stratum by a layer, half a metre thick, of archaeologically sterile deposits. It seems unlikely that three samples could have separately moved up in stratigraphic order from the underlying Middle Palaeolithic layer; their small size made it possible to eliminate many possible contaminants that could have leached from above, and use of the accelerator technique obviates the lumping of many samples, sometimes from considerable stratigraphic thickness, often required by conventional radiocarbon dating. The National Science Foundation accelerator facility at the University of Arizona dated the samples, which were prepared by Bischoff at the US Geological Survey.

The article by Cabrera and Bischoff accompanies a second paper, on L'Arbreda cave, by Bischoff and Narcis Soler and Julià Maroto (both archaeologists) and Ramón Julià (a geologist). At L'Arbreda in Gerona (Catalonia) a long Middle Palaeolithic sequence underlies a series of Upper Palaeolithic deposits, the lowest of which is assigned to the early Aurignacian. Bischoff obtained three dates on charcoal from a 5-centimetre thickness of the uppermost Mousterian level: 39.4 ± 1.4 , 34.1 ± 0.75 and 41.4 ± 1.6 kyr BP. From a 5-centimetre thickness of the basal Aurignacian level he obtained four dates ranging from 39.9 to 37.7 kyr BP, each with a standard deviation of about

$\pm 1,000$ years. There is no sterile deposit between the two cultural levels; their sedimentary matrices are identical and there is no indication of a hiatus or lacuna. The differences observed archaeologically are ones of typology, artefact manufacturing techniques and lithic raw materials. The conclusion that the transition from Middle to Upper Palaeolithic in Catalonia was swift is supported by Bischoff's 20 stratigraphically coherent uranium-series dates on moss-generated carbonates interstratified with Mousterian levels in the site of Abric Romaní, 60 kilometres south of L'Arbreda near Barcelona¹³. These dates span the period between 60,000 and 40,000 years ago and confirm that the Middle Palaeolithic technology terminated here at about 40 kyr BP. Other dates for the end of the Mousterian in Spain range between 37 and 39 kyr BP.

Assuming that all the El Castillo and L'Arbreda samples had not been moved upward from earlier levels, the 40,000-year-old dating of the Aurignacian at these two northern Spanish sites has a number of implications that can be pro-

posed as a series of hypotheses, not all of which are mutually exclusive:

- The Aurignacian does not represent an actual migration of people, but rather a simultaneous technological development, largely the product of convergence. Such a hypothesis would be in concordance with the minority view that European Neanderthals evolved *in situ* into Cro Magnon with only some gene flow from Asian and African populations of anatomically modern humans.

- Mousterian-derived Chatelperronian and new, 'intrusive' Aurignacian technologies overlapped for a substantial amount of time in south-west Europe (about 5–7 kyr), suggesting a significant chance for cultural and genetic interaction.

- The migration of modern people with Aurignacian artefacts from south-east to south-west Europe was extremely fast.

- Earlier dates — beyond the limit of the radiocarbon technique, which is close to 40 kyr — may yet be obtained from southeastern and central Europe, especially by thermoluminescence.

- The conventional radiocarbon and palynological dates for the late Mousterian and Chatelperronian may be all too young *vis-à-vis* these accelerator dates for the early Aurignacian.

Palaeoanthropologists are not yet in a position to choose definitely among these and other possible hypotheses, but Bischoff's work opens up the debate to a wide variety of possibilities. It has already

added much detailed chronological information to the rich Palaeolithic record from northern Spain¹⁴. Even for such a recent period as the Middle–Upper Palaeolithic transition, a great deal remains to be learned. In the next few years we are likely to witness exciting research to try to date and explain the disappearance of the Neanderthal morphology and the development of the sophisticated Upper Palaeolithic technologies, art-forms and strategies for survival. □

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manent change in volume accompanying chemical differentiation of the deep interior can at least partially offset the effect of thermal contraction from a hot initial state. The key to their argument is partial melting of a deep-mantle layer containing aluminous silicates. At pressures in excess of about 2 gigapascals, the aluminous phase will be the comparatively dense mineral garnet. Partial melting of such mantle material will yield buoyant magmas that should rise to much shallower levels where the crystallizing aluminous phase will be of significantly lower density. The combined volume of the crystallized magma and mantle residue is less dense by an amount that depends on the starting composition and the degree of partial melting, but which could be as much as 2–5 per cent for possible lunar bulk-composition models. Such differentiation-induced density changes have been invoked to explain the stabilization of thickened continental lithosphere on Earth⁹ and the support of the elevated topography of the Tharsis Rise volcanic province on Mars¹⁰.

Kirk and Stevenson have calculated the time-dependent volume of the Moon for a suite of thermal-history models that include the effect of differentiation-induced changes in volume. Their models presume that heat transport at temperatures below the solidus is conductive. This is partly to provide a comparison with earlier such models² in which the effect of differentiation was ignored and partly because there is no straightforward scheme for incorporating differentiation into a convective thermal-evolution calculation. The important differentiation process is the partial melting of mantle material deeper than 400–500 km (that is, within the garnet stability field) and upward transport of the magma so produced to the upper 200 km (within the

LUNAR GEOLOGY

Ironing out the wrinkles

Sean C. Solomon

THE giant-impact hypothesis for the origin of the Moon, although gaining widespread support, has a persistent problem. The hypothesis predicts complete melting of the source material of the Moon, yet the lunar tectonic record is inconsistent with the cooling and contraction that an initially molten Moon would have experienced. Kirk and Stevenson¹ now demonstrate that consideration of the chemical differentiation of the lunar mantle may help to resolve this problem.

According to the giant-impact hypothesis, the Moon formed from an accretion disk thrown into circumterrestrial orbit after the collision of a Mars-size object with the Earth^{2,3}. Numerical models of the impact event⁴ and of the evolution of the accretion disk⁵ suggest that the lunar source material underwent complete melting. The changes in global volume that would accompany cooling from such an initial state, however, should have led to the formation of large-scale tectonic features⁶, which are not observed. If thermal expansion and contraction are

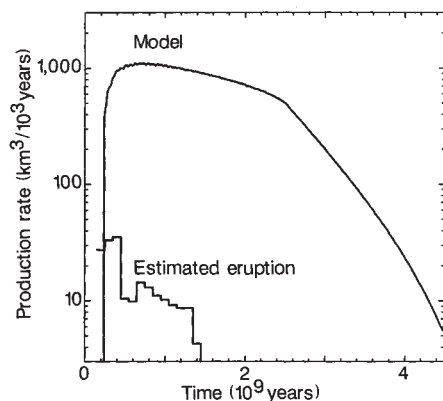
the dominant sources of global volume change, models of lunar thermal history that are consistent with a nearly constant volume since the end of heavy impact bombardment 3,800 million years ago indicate that only the outer 200–400 km of the Moon were initially molten⁷. In such models, the initially molten outer layer — or magma ocean — differentiated early to form the lunar crust and the source regions of lunar mare basalts⁸, while the warming and expansion of the initially cold interior served to offset the cooling and contraction of the outer layers so as to maintain a nearly constant lunar radius over subsequent lunar history.

Kirk and Stevenson¹ argue that thermal expansion is only part of the story of lunar volume. In particular, a per-



The pitted lunar surface shows no signs of tectonic activity.

From The Planetary System, Addison Wesley



Rate of magma production in the deep mantle of the Moon versus time since lunar formation¹. Shown for comparison is the estimated rate of volcanic eruption on the lunar surface⁹.

feldspar stability field). Kirk and Stevenson find that their models that satisfy the constraint on nearly constant lunar volume in the past 3,800 million years can be initially molten to as great as 500-km depth and can have a deep interior initially as warm as 1,200 K. The rate of production of magma from the deep mantle in one of their thermal history models is shown in the accompanying figure.

The extent and timing of lunar differentiation in Kirk and Stevenson's models have several unusual characteristics. The total volume of magma produced at depths within the garnet stability field is about 10 per cent of the Moon's, a figure comparable to the volume of the present lunar crust and approximately two orders of magnitude larger than the known volume of mare basalt volcanically erupted onto the lunar surface¹¹. Further, the models predict that this deeply derived magma has been produced continuously since the end of heavy bombardment (see figure).

Because most of the lunar crust is thought to have been emplaced substantially before the end of heavy bombardment⁸, Kirk and Stevenson argue that the bulk of this deeply derived magma solidified within the shallow subcrustal mantle. And because this magma was derived

from the initially solid deep interior, it need not bear any geochemical signature of the early differentiation of the lunar magma ocean, as the mare basalt source regions do⁶. Kirk and Stevenson argue that this history is consistent with the known geophysical properties of the lunar mantle. It is not clear whether any basalts that originated by such partial melting of the deep mantle, perhaps to be modified later by shallow fractionation or crustal assimilation processes, are present in the collection of Apollo or Luna samples, but this is not a requirement of their models.

One of the consequences of Kirk and Stevenson's work is that the possible initial states of the Moon consistent with the lunar tectonic record now include generally hotter models. Whole-Moon melting is still precluded, however, because the early differentiation of an initially molten Moon would not be expected to leave a

deep aluminous mantle capable of yielding global volume changes upon later partial melting. Kirk and Stevenson speculate that the Moon may have formed from the aggregation in Earth orbit of a number of hot but partially frozen moonlets, with the denser chilled portions of the moonlets sinking to form an initially solid 'core' in the early Moon. But quantitative elucidation of this idea must await further work.

A more general result of Kirk and Stevenson's study is that global differentiation must be considered in any analysis of the tectonic evolution of a one-plate planet. Because of its history of extensive volcanism and widespread faulting, a leading candidate for such an analysis in the future is Mars. □

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IMMUNOLOGY

Stimulating killer cells

Michael J. Bevan

CYTOTOXIC T lymphocytes (CTL) provide an effective defence against many intracellular pathogens — in some cases full protection can be achieved even in the absence of an antibody response¹. Unlike antibodies, which bind foreign proteins in their native form, T cells recognize foreign proteins as short fragments bound to cell-surface molecules encoded by the major histocompatibility complex (MHC). It was therefore reasonable to hope that synthetic peptides, which are able to mimic naturally processed antigen in T-cell recognition assays *in vitro*, might also be used as effective T-cell immunogens *in vivo*. Most experiments have indicated otherwise, however. Although peptide immunization can stimulate MHC class II-restricted T cells to 'help' B cells secrete antibody, MHC class I-restricted cytotoxic T-cell (killer cell) responses are rarely induced. Given these past failures, the success reported on page 561 of this issue is significant. Deres *et al.*² show that adding a lipoprotein tail to peptides is a reproducible — and probably generally applicable — way of stimulating a primary CTL response *in vivo*.

It is not known how the lipid attachment, a synthetic analogue of the active moiety of a major lipoprotein of *Escherichia coli*, converts peptides into *in vivo* immunogens for CTL. The conversion may simply be due to a longer half-life of the peptide or the known mitogenic properties of the moiety may be the key. Alternatively, the most important consequence of the modification may be to deliver the peptide to the cytoplasm where it can enter the normal pathway of antigen

presentation that leads to association with class I molecules of the MHC. The ability to insert themselves into membranes seems to be a feature of both the few unmodified peptides that have been shown to prime CTL responses *in vivo*³, and of the lipoprotein tail. Although experiments using fixed cells show that efficient access to this pathway is not required for the presentation of peptides to primed CTL *in vitro*, the concentration of circulating peptides may not be maintained at a high enough level for this type of presentation to be effective *in vivo*.

Although the reasons for Deres *et al.*'s results have not been conclusively established, it is clear that a threshold of antigen-presenting efficiency has been crossed. This threshold appears to be higher for class I-restricted than class II-restricted presentation. I would argue that this is a consequence of class-specific differences in the intracellular pathways by which MHC-peptide complexes are formed.

Recent evidence indicates that class I molecules bind peptide deep within the cell, as they are being assembled in the endoplasmic reticulum⁴. Any protein that is endogenously synthesized, or is present in the cytoplasm, is able to supply peptides to this pathway of presentation. Although this means the pathway provides a way of sampling the internal components of the cell for immunosurveillance by CTL, it also means that any foreign proteins have to compete with more than 10,000 species of self proteins for presentation at the cell surface. Proteins synthesized on free ribosomes compete for transport from the cytosol into the endoplasmic reticulum as

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The colourful behaviour of garter snakes

AMONG other things, evolutionary biologists dream of finding a full explanation for the high levels of genetic diversity found in natural populations. One popular idea is that such variation might be related to the complexity of the environment. According to theory, the conditions under which environmental heterogeneity might favour the maintenance of genetic variability are likely to be less restrictive if individuals select those niches or habitats in which they are fittest; in other words, perhaps genes can 'choose' habitats.

In the case of defence against predators, it has been suggested that selection might favour particular combinations of colour pattern and behaviour leading to a build up of genetic correlations between the two. Many studies have been carried out to try to find evidence for such correlations between behaviour and morphology; some have apparently been successful, though few of them have been claimed to demonstrate a genetic basis.

On page 542 of this issue, Edmund Brodie reports the existence of genetic correlations between morphology and antipredator behaviour in natural populations of the garter snake (*Thamnophis ordinoides*) in coastal Oregon. This species is highly polymorphic with regard to colour pattern (see figure). Individuals also differ in their behavioural response to predators in ways which include sprint speed, endurance, antipredator display and the number of reversals during flight. Brodie has shown that these behaviours are not mutually exclusive but that the degree to which they are



present in an individual depends to an extent on its colour-pattern phenotype.

This correlation does indeed seem to have a genetic basis. But it is not clear whether it results from pleiotropy (genes with multiple effects) or linkage dis-

equilibrium (the nonrandom association of alleles at different loci), although Brodie favours the latter. It will be fascinating to see which one of these alternatives is supported by future research.

Rory Howlett

well as for binding to class I MHC. This degree of competition is unique to the class I-restricted killer system and may explain why epitopes (peptide fragments) able to stimulate class I-restricted CTL are found less frequently than those able to induce class II-restricted T cells. It may also explain why efficient delivery to the cytoplasm is vital if peptides are to be effective CTL immunogens *in vivo*.

Neither access to the normal pathway of presentation nor competition with other peptides presents such formidable hurdles to the induction of a class II-restricted response. Class II molecules specialize in presenting peptides derived from antigens that come from outside the cell, and endocytosed peptides or proteins readily gain access to the compartment in which association takes place. Unlike the class I pathway, where there are no obvious cues to distinguish self from foreign proteins inside the cell, selective uptake of antibody-bound or aggregated antigen may serve to concentrate foreign antigens relative to self proteins in the endosomes, and competition for class II association is thereby avoided, at least to some extent.

Because so many proteins compete on

an equal footing for class I presentation, epitopes able to stimulate CTL responses may be present in only a few antigenic complexes at the surface of an infected cell. Only CTL with high-affinity receptors will be able to respond, a point that is of some relevance to vaccinating with peptides. An acid test for the physiological relevance of killer cells that have been raised by peptide immunization is their ability to lyse target cells expressing the natural antigen endogenously. Unlike the peptide-specific CTL that can be induced by using high concentrations of peptide in culture⁵, the effector cells described by Deres *et al.* pass this test.

In spite of the promise of this approach, the rarity of physiologically relevant class I-restricted epitopes raises questions over the usefulness of attempting immunization with peptides. The problem of infrequent epitopes is further compounded by the polymorphism of MHC molecules, which means that each binds a different range of peptides. If an immunogen as complex and foreign as influenza cannot be presented by some class I alleles⁶, peptide vaccines will have to be carefully tailored to individual MHC alleles. This

might not be completely impractical, as some class I alleles occur frequently in the population. Even so the rarity of epitopes is such that non-responsiveness is likely to occur if epitopes from only a single protein were used. For example, when hen ovalbumin is presented as an endogenous antigen, H-2^d and H-2^k mice fail to mount a CTL response⁷. It may be possible to provide a useful level of protection in an outbred population with peptide vaccines. But to achieve this a carefully designed cocktail of peptide epitopes, derived from all subunits of the pathogen, would probably have to be used. □

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Testing Fermi-liquid models

G.A. Sawatzky

In trying to identify the mechanism responsible for the behaviour of high-temperature (high- T_c) superconductors, it is important to obtain a useful description of the electrons in the materials, above and below the transition temperature, especially near the characteristic energy or 'Fermi level'. Several groups¹⁻³ have reported high-resolution angle-resolved photoelectron spectroscopy

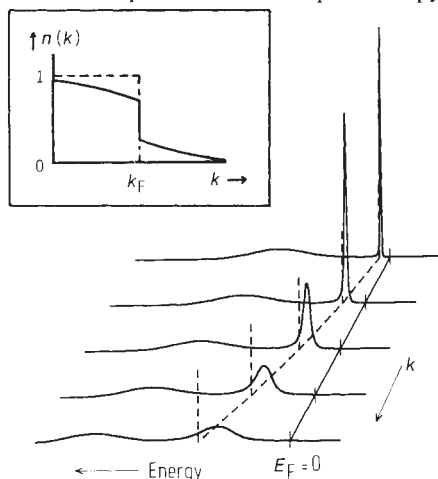


FIG. 1 In angle-resolved photoelectron spectroscopy (ARPES), both energy and momentum conservation must be considered: the emitted electron, excited by a photon of fixed energy, necessarily has momentum opposite to that of the electrons remaining in the sample. This restricts the experiment to exciting only a narrow set of energy states. Changing the angle at which electrons are detected, changes the resonance energy. Broken lines, spectra for a one-electron Fermi liquid; solid line, an interacting-electron system describable as a Fermi liquid. Inset, occupation of momentum states for one-electron system (broken line) and real system (solid line) showing the discontinuous jump at the Fermi momentum.

(ARPES) data purporting to indicate 'Fermi-liquid' type behaviour — that is, the effective charge carriers move independently. It is worth taking a closer look at these data and at what the technique can really show.

The ARPES technique is used to measure the probability that the material in its ground state of, say, N electrons can be raised by a monochromatic photon into a final state of $N-1$ electrons and a free electron, which is detected. From this one can determine the character of the valence band in which the N or $N-1$ electrons are found and which is responsible for the electronic properties of the material. If the photon energy is sufficiently high, the free electron departs from the material without interacting with the other electrons and the simplifying 'sudden' approximation can be used

to relate the electron spectrum to the states of the material.

In the most simple model of electron behaviour, the one-electron model, each electron in the valence band is assumed to move independently. A primary characteristic of such a 'Fermi liquid' of electrons is that all possible electronic states below the Fermi level and corresponding Fermi momentum are filled and all those above are empty (Fig. 1, inset). The ARPES spectrum for such a one-electron system would consist of a single peak the energy of which varies with the detection angle (related to the electron wavelength or momentum) and which vanishes when pushed above the Fermi level, which corresponds to a well defined momentum in the solid (Fig. 1).

In real materials, there are interactions between the electrons, of the electrons with the lattice vibrations (phonons) and, in magnetic systems such as (perhaps) the high- T_c superconductors, of electrons with 'magnons'. For the Fermi-liquid description still to apply⁴, it is not possible to discuss simple electrons. Instead, it is necessary to define new quasiparticles corresponding to the electrons 'dressed' with a distortion of their surroundings due to the various interactions.

With the correct description, one finds that all states below the Fermi level are occupied by quasiparticles, all those above are empty. The lifetime of the quasiparticle at the Fermi level must be infinite, which leaves its imprint on the ARPES spectrum as a peak at the Fermi energy with no width (Fig. 1). For the description to be useful, the properties of the quasiparticles must change smoothly with small changes of energy around the Fermi level. This implies that the width of the spectral peak changes smoothly, say

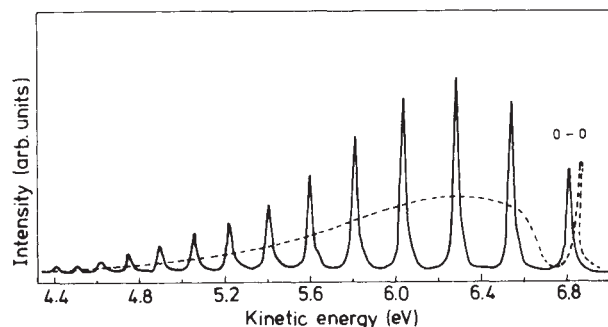
as the square of the difference, for departures from the Fermi energy (Fig. 1).

The ARPES spectrum of the quasiparticle Fermi liquid is particularly interesting (Fig. 1). There is a small additional angle-dependent shift of the peak energy related to the fact that the quasiparticle's mass is greater than that of the simple electron. The energy-dependent broadening yields the particle's lifetime. The structure that arises at higher energy is significant as it is related to the interactions that give the quasiparticle its character (Fig. 2): the sudden removal of an electron from its dressing leaves the remaining system in various higher states corresponding to excitations of phonons (lattice vibrations), plasmons (current vortices), magnons (spin states) and so on. The number and distribution of these reveals what the dressing was made of. Because the excitations detract from the intensity of the lowest-energy peak, the integrated intensity of the latter is a second indicator of the quasiparticle mass.

Thus, to demonstrate that the high- T_c superconductors can be described in terms of a Fermi liquid (at least, above their transition temperature) it is necessary to show that the ARPES spectrum includes a sharp peak corresponding to the quasiparticle, well separated from a broad, incoherent continuum corresponding to the broadened excitations. The width of the quasiparticle peak, and its derivative with respect to energy, should tend to zero as the detection angle is changed to tune the peak to the Fermi level and corresponding momentum. And the peak should vanish for energies above the Fermi level. Of course, finite temperature and finite energy resolution weaken the precision of these criteria.

The published spectra do display an angle-dependent feature ('peak' seems too strong a description). The extent to which it moves with changing angle is about a tenth of that predicted by theory,

FIG. 2 Schematic photoelectron spectrum of gaseous hydrogen⁵ (photon energy, 21.2 eV). Insofar as the vibrational progression of peaks is due to the bonding character of the H_2 1s electrons, the equilibrium bond length is dependent on the degree of occupation of that level. The electrons are dressed by interatomic displacements. The intensities are given by Franck-Condon factors, the molecular equivalent of the sudden approximation. The ARPES spectrum of solid hydrogen, developed from the molecular spectrum, will be angle dependent, but for some angle will resemble the broken line. The fundamental transition (0-0) becomes the solid-state quasiparticle peak. The phonon excitations develop into a broad, incoherent quasicontinuum. For a Fermi liquid, the two features should be clearly distinguishable. The relative intensity of the 0-0 peak, can be related to the quasiparticle mass: for a well defined quasiparticle, electron and dressing move coherently.



which would seem to indicate that about 90 per cent of the spectral intensity should be found in the incoherent continuum and that the effective mass is ten times that of the electron. Indeed, each group finds a very large 'background' to the spectrum. But in none of the spectra is there a clear separation of the peak from the background. In fact, the feature's structure typically is rather broad and strongly asymmetric, the shape changing as rapidly with changing angle as does the feature's maximum, if not more rapidly. Furthermore, the point at which the Fermi level is crossed is not all that clear.

These observations indicate a system in which the electrons are highly dressed and in which interactions between quasiparticles have an important effect, in particular on the asymmetric spectral structure. Nevertheless, the observation of a Fermi level (a sharp energy cut-off) does indicate that we are dealing with Fermi-type particles (dressed electrons). Theories based on Bose-type particles, such as electron pairs bound by magnetic or lattice interactions, would be hard pressed to account for this fact. A gap of 30 meV appears in the ARPES spectrum

as the superconductor is cooled through its transition temperature, corresponding to the gap predicted in the 'BCS' theory of conventional superconductivity.

Despite all this, the data do not persuade me that it will be fruitful to concentrate on Fermi-liquid theories for describing the normal state or the transition to the superconducting state of the oxide superconductors. Even higher resolution is required to show whether well defined quasiparticles dominate the spectrum within 30 meV of the Fermi level. If they do, one can turn to Fermi-liquid models. But if it becomes clear that the incoherent part of the spectrum extends within this range of the Fermi level or if the coherent peak fails to behave properly, theorists will have to look to other models. □

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NEUROETHOLOGY

Deceptively simple behaviour

Jennifer Altman

INVERTEBRATES and lower vertebrates supply neurobiologists with many excellent opportunities for studying the relationship between nervous systems and behaviour. The choice of these animals for research has rested on two assumptions: that they have relatively simple nervous systems, and that their behaviours are stereotyped — that is, a particular response is always carried out in the same way. The first assumption has seemed increasingly shaky in the past few years, as what were thought of as simple linear circuits are now recognized as complex distributed networks. The picture that emerged from two recent meetings* means that the second assumption must also be called into question — the behaviour of lower animals has variability and flexibility very like that seen in higher vertebrates.

Here are a few of the many examples on offer. Careful examination of the development of various forms of simple learning in the gill-withdrawal reflex in juveniles of the sea-slug *Aplysia* is forcing a revision of ideas about the organization of the learning processes in the adult (T. Carew, Yale

University). Gill withdrawal is a simple protective response that has been used for many years to study the cellular basis of synaptic plasticity¹. If the siphon is touched repeatedly, the response wanes or habituates but a novel stimulus at another site produces dishabituation — the full response returns. A third form of learning is sensitization; the response increases when a noxious stimulus, such as an electrical shock to the animal's tail, is given before the reflex habituates.

As dishabituation and sensitization both involve a facilitation of the response, they have generally been thought of as different manifestations of the same process; most studies have thus been carried out on dishabituation because it is easier to control. But Carew and colleagues have found striking differences between the two processes; for example, during development dishabituation appears about 60 days before sensitization. Looking carefully again at adult *Aplysia*, they have found that here too dishabituation and sensitization can be separated. The processes are differentially sensitive to stimulus strength and occur at distinctly different times after stimulation².

This separation has also led Carew's group to re-examine the cellular basis of the learning processes. They conclude that, in addition to the much-studied

monosynaptic connection between the sensory and motor neurons, a polysynaptic pathway of as yet unidentified interneurons seems to be necessary to explain all the forms of plasticity seen in the reflex. So *Aplysia* begins to come into line with higher animals, where learning takes place not just in reflex circuits but also in pathways parallel to them.

Rapid escape manoeuvres, such as the evasive running of cockroaches and crickets, have long been thought to epitomize stereotyped responses. More detailed examination of the behaviour in cockroaches has, however, revealed considerable variability in the direction (R. Ritzmann, Case Western Reserve University, Cleveland) and the duration (H. Gras *et al.*, Universität Göttingen) of the run. In cockroaches, the behaviour can be divided into an initial turn away from the stimulus, lasting about 60 milliseconds, followed by a run in a random direction that takes the animal out of danger. The cockroach is responding to wind currents — of the sort that would be generated by a predator approaching from behind — that are detected by appendages on the abdomen. Evasive running is suppressed in female crickets during mating, when the male also comes from behind (F. Huber, Max-Planck Institut, Seewiesen). Presumably the species is saved from extinction by changes in the coupling between neurons in the evasion pathway, possibly brought about by alterations in the concentration of a sex hormone.

The problem of adapting the output of a circuit to the demands of the moment has been much studied in the stomatogastric system — the small group of neurons that controls stomach movements in lobsters and crabs³. Recently, Heinzel and Selverston⁴ provided behavioural evidence that neuromodulatory chemicals can alter the interactions between neurons and thus change the output of a network. They observed the movements of the teeth in the lobster's stomach as they injected the pentapeptide proctolin into the animal; when proctolin is present, the movements of the teeth change from grinding to chewing⁵.

In the stomatogastric system, the organization of the networks that control the four parts of the stomach are now known to be influenced by at least ten neuromodulatory transmitters, which work either by changing intrinsic membrane properties or by varying the strengths of synaptic coupling (E. Marder, Brandeis University; see the recent News and Views article by Selverston⁶). An important problem for the future is how the complex effects of so many neuromodulators are coordinated to prevent networks becoming over-modulated to the point where they cease to function (Marder).

Another outstanding question, now

* Second International Congress of Neuroethology (ICN), West Berlin, 10–16 September 1989; satellite symposium Motor Control in Arthropods, 18–21 September, Tutzing, West Germany. The proceedings of the second ICN are published as *Neural Mechanisms of Behavior* (Thieme, Stuttgart).

that neuromodulators are known to influence the expression of particular behaviours, is what controls the modulatory neurons — to answer this, further studies of the circumstances that elicit the behaviours are needed (E. Kravitz, Harvard University).

Networks like the stomatogastric system that can generate more than one output have been termed 'polymorphic', although 'polyfunctional' would be more accurate. Getting⁷ first introduced the concept to describe the network controlling both withdrawal and escape swimming in the giant sea-slug *Tritonia*. Generally, it has considerable heuristic value but the original meaning is already being blurred. Strictly, a polymorphic network signifies a change not just in output pattern but also in the operations within the network (P. Dickinson, Bowdoin College, Maine). The generation of chirps and frequency shifts in the signals of electric fish (W. Heiligenberg, University of California, San Diego) also seems to conform to the strict definition.

In several other cases, polymorphic networks may exist, including ecdysis movements in larval and pupal stages of the moth *Manduca sexta* (K. Mesce, University of Minnesota), the scratch reflex in turtles (P. Stein, Washington University, St Louis), and walking, kicking and scratching leg movements in chicks (A. Bekoff, University of Colorado, Boulder). The polymorphic concept should influence the way that neuronal circuits underlying such behaviours are investigated in future.

All the variability and flexibility that is becoming apparent as behaviours are subjected to close scrutiny should be seen as a challenge rather than a nuisance hampering research (C. Gerhardt, University of Missouri). Variability and flexibility are the raw material of adaptation. They enable animals to cope with the demands of the environment and provide investigators with a powerful tool for analysing the constraints on neural organization. The challenge now, as Carew succinctly put it, is to dissect these complex systems and learn to put them back together again, keeping firmly in view their functions in the lives of their owners. □

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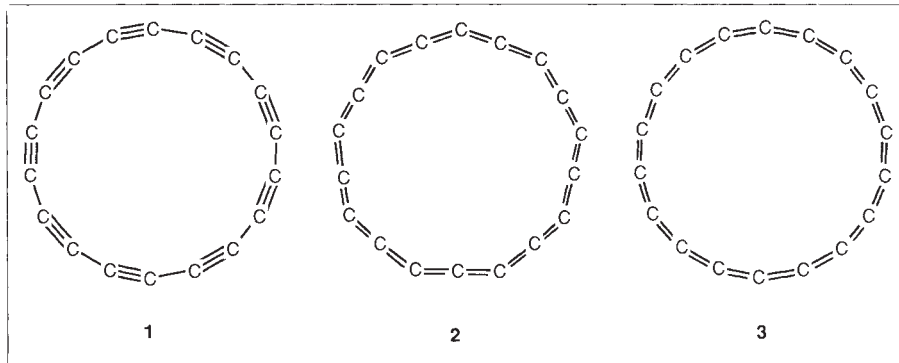
Towards the cyclo[*n*]carbons

Fraser Stoddart

THE quest to synthesize unnatural products¹ gets stronger with time. Incentives are many and different. One is the feeling — often spurred on by growing speculation and healthy discussion — that something which has not been done is just waiting to be done. Only it takes some daring to do it. Despite our long and unique fascination with diamonds and the very many applications we have found for graphite, no discrete and homogeneous all-carbon compounds have so far been prepared by total synthesis using a rational approach. But the report in *Science* of a synthetic approach to the cyclic molecule C_{18} (structure 1) by Fran-

systems of π -orbits, one in-plane and one out-of-plane, giving the molecule a distinctive form of aromaticity.

Semiempirical and *ab initio* quantum mechanical calculations for C_{18} incorporating appropriate geometry optimizations lead to two conclusions of some significance. First, the three cyclic structures 1, 2 and 3 are all considerably more stable than the singlet acyclic structural analogue. And second, of these three, structure 1, with its alternating single and triple bonds, has a lot lower energy than the other structure with 9-fold rotational symmetry (structure 2) or the more highly symmetrical 18-fold structure 3.

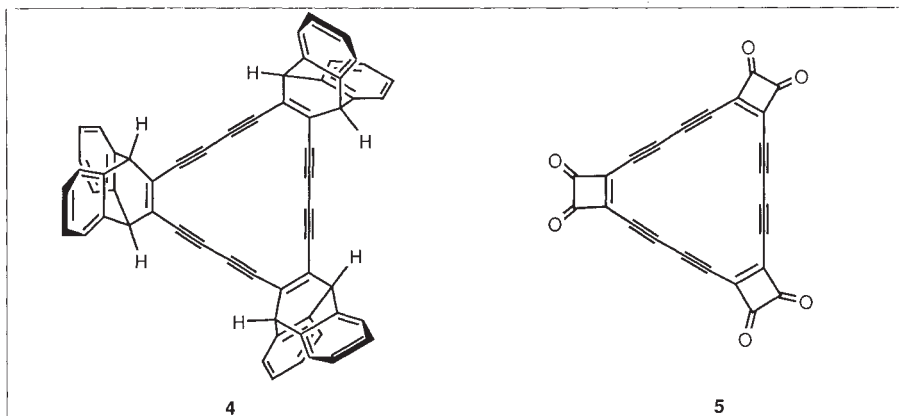


çois Diederich and colleagues² looks set to change this situation and break new ground in the chemistry of all-carbon compounds.

In recent years, C_n molecules³ in general — and in particular the soccerball-like C_{60} compounds⁴, referred to variously as buckminsterfullerene and footballene, which are claimed to be formed when graphite is vaporized by laser irradiation⁵ — have caught the imaginations of theorists^{6,7}. The general consensus is that, in the range C_{10} to C_{20} , monocyclic ring structures are preferred to cage-like structures (like C_{60}) or linear ones. Neutral cycles with $4n+2$ atoms are especially stable; for example, cyclo[18]carbon (structure 1), which Diederich points out can be stabilized by two perpendicular

Moreover, because the estimated strain energy of 72 kilocalories per mole based on about 4 kcal mol⁻¹ for the distortion of each of the 18 bond angles from 180° to 160°, is much less than the dissociation energy of about 130 kcal mol⁻¹ required to cleave a C—C bond joining to acetylenic units, the synthesis of compound 1 seemed feasible.

To synthesize compound 1, Diederich *et al.* identified and prepared^{2,8} appropriate cyclic compounds based around an 18-carbon-atom ring, the dehydro[18]-annulenes such as compounds 4 and 5. These can be synthesized from readily available starting materials. In each case, the authors supposed, the functional groups at the vertices could be cleaved from the C_{18} ring by irradiation or flash



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vacuum pyrolysis. In the case of compound 5, this would occur by the extrusion of carbon monoxide; and for compound 4, by the loss of three anthracenes by a reverse 'Diels-Alder' reaction.

In the event, the authors chose compound 4, whose structure they confirmed by X-ray crystallography. In contrast with its parent compound 1,3,7,9,13,15-hexa-dehydro[18]annulene ($C_{18}H_6$), which explodes at 85 °C, compound 4 is very stable. Nevertheless, flash-heating experiments, using 10^{-8} second ultraviolet laser pulses irradiating a thin film of compound 4 coated on a tantalum rod, did generate neutral desorption products whose mass spectrum measured in a time-of-flight mass spectrometer, indicated the presence of traces of C_{18} .

This observation suggests that it should be possible to isolate sizeable quantities

of cyclo[18]carbon (compound 1) by flash-vacuum-pyrolysis. When this happens then not only will it be 'a first' in carbon chemistry but these novel carbon allotropes could also serve, so Diederich suggests (personal communication), as precursors to new two-dimensional network polymers. □

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EMBRYOLOGY

Under arrest in the cell cycle

Tim Hunt

MATURE frog eggs contain powerful regulators of the cell cycle. These cells are arrested in metaphase, not a common place for the cycle to pause, though for frog eggs it is an appropriate one in which to receive a sperm. The question is, what stops the eggs entering anaphase, and how does the sperm break the spell? According to two papers^{1,2} in this issue of *Nature* (pages 505 and 512), the surprising answer is an oncogene, *c-mos*, whose product pp39^{mos} is destroyed on fertilization.

The story starts in 1971, when Masui and Markert³ discovered that microinjection of frog-egg cytoplasm into a fertilized egg stopped the recipient cell dividing and arrested it stably in mitotic metaphase. Because the recipient cell is arrested in the same stage of the cell cycle as the donor cell, they suggested that the metaphase-arrested state of eggs can be ascribed to a special factor they called 'cytostatic factor' or CSF for short. Attempts to purify and characterize CSF over the intervening years have been frustrated by its low abundance and tendency to inactivate — it was known to be exquisitely sensitive to calcium, which destroyed it⁴.

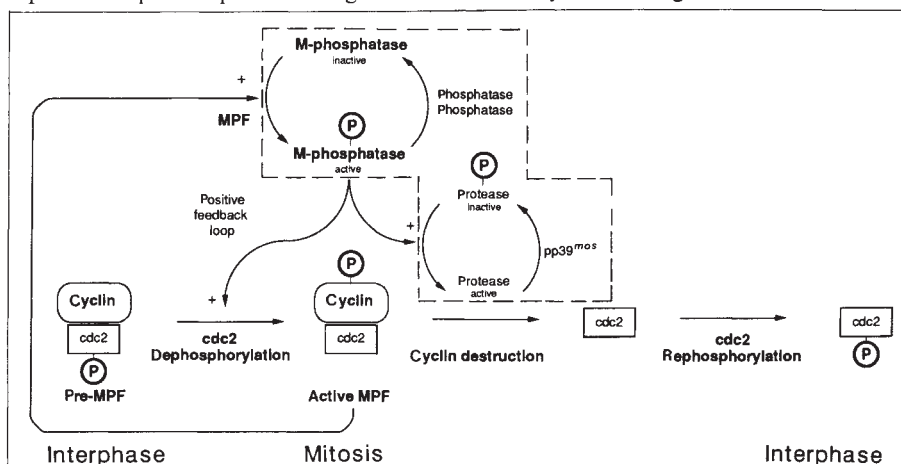
The characterization of the other cell-cycle regulator from frog eggs, maturation-promoting factor (MPF), has been more successful⁵. MPF induces cells to enter meiosis or mitosis, but does not hold them there. Traditionally assayed by injection into G2-arrested frog oocytes, MPF is itself very unstable, hard to purify and sensitive to calcium. MPF is now known to be a complex of a p34^{cdc2} protein kinase subunit together with a cyclin^{6,7}. Considering that the assay for MPF is injection of egg cytoplasm into oocytes, whereas the assay for CSF is injection of

the same starting material into cleavage-stage blastomeres, one could be forgiven for wondering if the same molecule(s) could be responsible for both effects, but this has never been thought to be true. Pure MPF does not possess CSF activity, and CSF does not possess MPF activity. In the light of the *c-mos* results this is understandable.

The *c-mos* proto-oncogene was recognized about ten years ago as the cellular homologue of the transforming gene from the Moloney murine sarcoma virus^{8,9}. It is a powerful species-specific oncogene that

requires expression of only a few copies per cell to cause transformation. In normal growth and development, mouse *c-mos* mRNA was detectable only in testis and oocytes^{10,11}, suggesting that it had a role in meiosis or early development. The data from Watanabe *et al.*² show that expression of the protein in *Xenopus* is dramatically restricted in time as well as in space. Although *c-mos* mRNA in oocytes is present from early in oogenesis and persists to gastrulation, pp39^{mos} begins to accumulate only when oocytes are exposed to progesterone, which induces maturation, and all the protein present in mature eggs is destroyed within 30 minutes of fertilization, never to reappear. This may be one of the most striking examples of developmental translational and post-translational control yet observed. The level of pp39^{mos} shows a short sharp spike that holds the unfertilized egg in M-phase until fertilization, when a calcium spike triggered by the successful sperm activates sudden protein destruction, mediated in part at least by a calcium-dependent protease called calpain II. The mRNA level stays constant, giving not the faintest clue of the behaviour or function of the protein it specifies.

What is the evidence that pp39^{mos} is what holds cells in M-phase? Sagata *et al.*¹ did two telling experiments. First, they injected *c-mos* mRNA into one of the two blastomeres of a recently fertilized *Xenopus* egg and found that the injected cell stopped dividing, arresting with condensed chromosomes typical of metaphase. Lower doses permitted one or two divisions before the arrest took hold, presumably reflecting the need for a



How *c-mos* can stabilize MPF. Starting at the left, pre-MPF consists of a complex of cyclin and phosphorylated p34^{cdc2}. It is activated by a phosphatase that can be activated by MPF (hence the positive feedback loop). However, active MPF also turns on cyclin protease, speculatively shown here as involving M-phosphatase so that pp39^{mos} can keep the protease turned off. Once cyclin is destroyed, p34^{cdc2} is rapidly rephosphorylated and thereby turned off until fresh cyclin is made. It is important to turn off the protease at the end of mitosis, or cyclin can never reaccumulate. According to the model, the protease is only active while the phosphatase is active, which in turn is only active as long as MPF is active. Essentially everything above the line of cyclin/cdc2 (boxed with dashed lines) is speculative, while everything on the line is well-documented. □

threshold level of the protein. Second, when CSF preparations from crushed frog eggs were depleted of pp39^{mos} with monoclonal antibodies, they lost their CSF activity. Control antibodies did not diminish the CSF content of the cell-free extracts. A similar conclusion can be drawn from recent experiments in mouse oocytes, where ablation of *c-mos* mRNA with antisense oligonucleotides prevented meiosis II arrest¹².

There is a close relationship between CSF and MPF despite their clearly different identities. CSF is in effect a specialized entity designed to stabilize MPF in metaphase-arrested eggs. CSF does not advance the cell cycle in microinjected *Xenopus* blastomeres; it simply locks the cell in metaphase once metaphase has been achieved by the action of MPF. The key question is therefore: how is MPF turned on and off, and how can its level be kept high? This issue was recently discussed by Andrew Murray in these pages¹³. The figure presents a speculative summary. There is no doubt that MPF is turned on by a protein phosphatase¹⁴ and turned off by a protease that digests its cyclin subunit. A key point is that cyclin is only unstable during mitosis, which means that MPF must itself activate the protease, probably indirectly as shown in the model. This provides a negative feedback loop that makes mitosis normally a self-limiting process. The circuit almost certainly contains a positive feedback loop as well; there are reasons to suspect that the phosphatase that turns on MPF is itself turned on by MPF as shown by the positive feedback arrow in the figure.

One interpretation of Sagata *et al.*'s results is that the putative cyclin-specific protease of the model is kept switched off by pp39^{mos}-mediated phosphorylation. According to this view, the protease is activated by a phosphatase, which by a simplifying guess could be the same phosphatase that activates and keeps MPF active. The idea that the cyclin protease is turned on and off by reversible phosphorylation is attractive, because it could explain why cyclin is only unstable during a narrow window that opens just before the onset of anaphase and closes once MPF activity has been lost. The alternative hypothesis is that cyclin itself can be made protease-resistant by *c-mos*-catalysed phosphorylation. The species specificity of *c-mos* might arise by co-evolution with the surprisingly variable amino-terminus of cyclin, which is required for destruction¹⁵ and which shows rapid evolutionary drift. It is not yet clear if cyclin is a substrate for pp39^{mos} and, if so, whether pp39^{mos}-phosphorylated cyclin resists destruction. This is of course an active area of investigation.

The puzzling variability of the cell-cycle arrest states shown by oocytes and eggs of different animals is now somewhat easier

to understand. Molluscan oocytes are fertilized directly in first meiotic prophase, frog and mouse eggs arrest in second meiotic metaphase, while sea-urchin eggs are blocked in the G1 phase of the first post-meiotic mitotic cell cycle. It used to be difficult to see how such variability could evolve without extensive changes to the cell-cycle control machinery, which as we now appreciate shows high evolutionary conservation. The *c-mos* results give an example of how this can be achieved; a protein kinase appears in the right place at the right time, phosphorylates a key cell-cycle regulator, and then vanishes.

How can *c-mos* act as an oncogene if its primary role is to block the cell cycle? The answer is not clear. One possibility is that pp39^{mos} indeed blocks somatic cells in metaphase, which may sometimes result in chromosome breakage and hence aneuploidy when cells escape the block. Another possibility is that *c-mos* could stabilize 'start' cyclins in the G1 phase of the cell cycle and thereby cause cells to proliferate inappropriately. Conceivably some sort of side reaction — for example the inappropriate phosphorylation of a protein quite unconnected with cell-cycle regulation — might lead to the transformed phenotype, but the effects of *c-mos* expression in cells will surely come under new scrutiny in relation to the cell cycle. It would be particularly interesting to know whether appropriate levels of pp39^{mos} expression block normal somatic cells in mitotic metaphase; was this the basis of its previously noted toxicity¹⁶? Paradoxically, the original finding that pp39^{mos} can promote frog oocyte maturation is now even harder to understand than it was at first report¹⁷. The new results make much more sense. □

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Frankenplastik

LAST week Daedalus decided that a polymer chain growing ionically in a magnetic field would grow in a circle, and close on itself to give a ring molecule. In strong solutions or liquid monomer, the growing rings will interlink to a sort of 'molecular chain-mail': a flexible solid with unique elastic properties. DREADCO chemists are now taking this idea further, by incorporating a few charged groups into the structure. Ideally, they would like each ring to have a positive charge and a negative charge diametrically opposite each other, and the whole structure plasticized for high molecular mobility.

The outcome should be a sort of plastic pseudo-electrolyte with loosely tethered ions. A current traversing the plastic will be carried by the charges, which will migrate like ions in an electrolyte. Stretched between its two rapidly separating charges, each ring will be pulled into a long thin loop; the plastic material will extend strongly. Reverse the current, and it will contract again. The long travel of the moving charges should absorb a great deal of electrical energy and convert it to mechanical movement.

So this novel plastic will act as a piezoelectric linear motor with an amazingly long throw. Like a conventional piezoelectric, it will also be a sensitive strain gauge. This elegant combined sensor and motor, silent and maintenance free, mouldable in any shape to deform in any desired way, and with the flexibility and feel of biological tissue, is clearly the perfect artificial muscle. DREADCO's Ionomuscle[®] should revolutionize robotics.

Already DREADCO anatomists and engineers are designing Ionomuscle robot arms, hands, legs, and so on, that mimic their human counterparts quite realistically. They won't have much advantage over conventional robot arms and actuators, though they should be very welcome in the artificial-limb market. But if DREADCO can manage to imitate the complexity of human musculature in close detail, the company will find itself facing a question straight out of science fiction. What use are truly humanoid robots?

Without a corresponding revolution in artificial eyes, ears and intelligence, Ionomuscle humanoid robots may look and feel quite lifelike but will be pretty untalented. Even so, their wide range of realistic preprogrammed poses and facial expressions should equip them for a variety of lucrative careers. Daedalus can see them employed as stunt men, television presenters, nondirective psychotherapists (equipped with a vocal version of the well known 'Eliza' programme), active shop-window dummies, decoy policemen, watchmen and security guards, and sexual surrogates.

David Jones

Ancient bone DNA amplified

SIR—Although the extraction and characterization of DNA from ancient soft-tissue remains is possible¹⁻³, it is the application of these techniques to the far more abundant remains of bone and teeth that will provide the opportunity for large-scale population sampling. We report the successful extraction and amplification of DNA from human bones between 300 and 5,500 years of age.

Typically, we can recover 5–10 µg of DNA from 2 g of powdered cortical bone. Although the DNA is degraded it is often possible to recover material of high molecular mass. Figure 1 shows DNA extracted from six well-preserved human bone samples after separation by agarose gel electrophoresis. Our results indicate that the preservation of DNA in a bone depends less on the age of the specimen than on the burial conditions of the skeleton.

We used the extracted DNA as a template for the polymerase chain reaction (PCR), which is ideal for amplifying DNA in biological samples with little or degraded DNA⁴. To monitor the amplification of contaminant DNA⁵ we performed control extractions and PCR, and sequenced fragments of the amplified DNA.

Some of the ancient DNA extracts, including the majority of the 19 seventeenth-century Abingdon bone samples examined, had an unknown contaminant that inhibited PCR, but inhibition was overcome in most cases by adding bovine serum albumin⁶. Our PCR amplifications were performed with only 2 units of Taq polymerase, as opposed to the 12.5 units of enzyme that was required for amplification of a DNA fragment from 7,000-year-old human brain tissue³.

Aliquots of 2–5% of the extract from 2 g of bone were used for PCR with pairs

of primers specific to various parts of the mitochondrial genome. Amplifications of 121-base-pair (bp) and 205-bp fragments of mitochondrial DNA were reproducible with extracts of different bones, and with different extracts of the same bone, including the 5,450-year-old specimen.

FIG. 2 PCR amplifications of mtDNA from ancient and modern DNA. Lane 1, DNA size markers in multiples of 123 bp; lanes 2–5, 205-bp fragment of the MTND4 gene obtained with primer 2 (see ref. 7) and primer 4 (mtDNA bases 11,211–11,230); lanes 6–9, 121-bp fragment obtained with primers A and B (see ref. 10). Templates: lanes 2 and 6, DNA from 750-year-old humerus; 3 and 7, DNA from 300-year-old femur; 4 and 8, modern DNA; 5 and 9, blank controls with no DNA.

METHODS. PCR amplifications were carried out by the method of Perkin Elmer Cetus in 25-µl reaction volumes, using 2 units recombinant Taq DNA polymerase per reaction. BSA (160 µg ml⁻¹) was added to overcome inhibitory activity (see text). For each reaction, 5 µl ancient-bone DNA were used, representing less than 5% of DNA extracted. Control reactions contained 1 ng modern DNA or no DNA, respectively. Each of the 40 cycles of PCR consisted of denaturation at 93 °C for 45 s, annealing at 55 °C for 30 s and extension at 72 °C for 60 s. Aliquots (10 µl) were electrophoresed on a 1.5% agarose gel (broad bands under the bands of interest are caused by excess primers). Direct sequencing from low-melt agarose¹¹ was performed with primer 4 and the Sequenase sequencing kit (US Biochemical Corp.).

Figure 2 shows these PCR fragments obtained from two of the ancient bone extracts and from modern human DNA.

The 205-bp fragment is part of a mitochondrial gene encoding the protein NADH dehydrogenase subunit 4 (MTND4). This fragment was amplified from one thirteenth and one seventeenth century bone extract and then sequenced directly from PCR. The two sequences were found to correspond to the published human mitochondrial sequence⁶ but with a C residue instead of a T residue at position 11,335, a common mtDNA variant⁷.

In a recent review⁸, it was stated that it is generally not possible to amplify ancient DNA fragments that are longer than about 150 bp, although well-preserved specimens may yield fragments of up to 500 bp. We amplified a 600-bp mtDNA fragment from the 750-year-old bone, but were unable to amplify a 1,100-bp fragment from ancient extracts (data not shown).

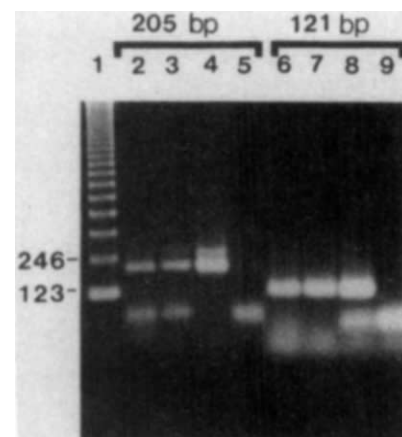
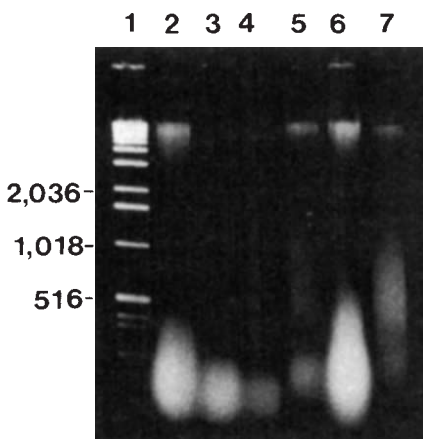


FIG. 1 Agarose gel electrophoresis of DNA extracted from bone samples of six individuals. Lane 1, DNA size markers; lanes 2, 3 and 4, DNA extracted from three femurs from a seventeenth-century English Civil War cemetery in Abingdon, Oxfordshire; lane 5, DNA from male humerus (radiocarbon date 750 ± 80 yr BP, Anglo Saxon-medieval cemetery, Abingdon; lane 6, DNA from the skull of a child (radiocarbon date 4,810 ± 80 yr BP, Maiden Castle, Dorset); and lane 7, DNA from a human femur (radiocarbon date about 5,450 yr BP, cave burial, Wadi Mamed, Judaeen Desert).

METHODS. The dirt and outside surfaces of the bone samples were removed by shot-blasting with fine aluminium oxide grit. The bone fragments were powdered in a Spex freezer mill refrigerated with liquid nitrogen, and then stored at -20 °C. After decalcification of 2 g powdered tissue with 0.5 M EDTA, pH 8.5, the DNA was extracted by the method of Maniatis *et al.*⁹ and concentrated by centrifugation-driven dialysis using Centricon 30 micro-concentrators (Amicon). DNA samples (10 µl), representing 5–10% of the DNA extracted, were loaded on a 1% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and photographed under ultraviolet light.



We have shown that it is possible to recover genetic information from ancient skeletal material if contamination by modern DNA is avoided. This work has significant implications for the study of past populations as it provides a source of primary evidence to add to the indirect evidence gained from modern population genetics, and from linguistic, cultural and anthropological sources.

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Folk physiology and talking hyoids

SIR—In the past it was generally believed that human speech derived solely from properties of the brain and that no special vocal anatomy was involved. Although much has changed, Marshall, as is evident from his recent *News and Views* article¹, has failed to follow this progress.

The issue in contention is whether Neanderthal speech ability was equivalent to ours, not whether they possessed speech and language. Marshall overlooks that we have consistently emphasized that they possessed these attributes²⁻⁴ and seems misinformed on even the basic theory of speech production — Muller's 'source-filter' model. The supralaryngeal vocal tract (SVT) acts as an acoustic filter letting peak energy through at 'formant frequencies' (refs 5 and 6), the main determinants of phonetic quality⁷. The filtering effects of the SVT are thus independent of the larynx. Marshall's comment that humans can produce 'intelligible' speech after surgical removal of the larynx is therefore irrelevant. As ape larynges are similar to ours with respect to sound-producing capabilities, we can assume that Neanderthal larynges were also similar. The debate therefore concerns the SVT, not specific laryngeal anatomy.

Marshall is in error concerning a number of points of speech anatomy and physiology. He incorrectly states that 11 muscles connect the larynx and hyoid when only one pair does — the thyrohyoids. Marshall also apparently believes that "... humans can learn to produce intelligible speech after surgical removal of the tongue ...". Talking or swallowing food normally is impossible when the tongue is removed. In speech pathology folklore there is a tale of a person talking with the entire tongue removed. Marshall seems to have swallowed the fable, confusing removal of the tongue 'body' (the main part of the tongue, extending into the throat) with the much less compromising removal of the tongue 'blade' (the protrudable part), although even this affects speech intelligibility. Marshall also asserts that "severe deformity of the vocal tract is compatible with human speech if a human brain is in command thereof." Many studies show that patients with normal brains and anomalous SVTs (such as palatal insufficiency, cleft palate) produce speech that is not as intelligible as normal speech owing to phonetic deficits, shown by acoustic analysis, psychoacoustic tests and computer modelling based on cephalometric X-rays⁸.

Marshall criticizes SVT computer-implemented modelling on the basis that the Neanderthal model did not account for laryngeal mobility. He overlooks that the model expressly gave Neanderthals the full modern range of laryngeal mobility⁹. He asserts that modelling inaccurately

predicts which vowels chimpanzees can make. Many studies have validated computer modelling for chimpanzees⁹, as well as normal human newborns¹⁰ and adults¹¹, humans with deformed vocal tracts⁸, and macaques⁵, and have affirmed that non-human primates cannot produce the 'point vowels' of human speech — [i], [u] and [a].

Marshall's claim that Jordan¹² showed that "real, live" chimpanzees produce [a] and [u] is not supported by the data. Instead of a sound spectrograph, Jordan used an octave-band analyser, with a limited high-frequency resolution, that reported formant bandwidths exceeding 1 kHz (vowel formant bandwidths range between 60 and 300 Hz). His spectra, therefore, cannot resolve the formant frequency patterns that differentiate vowels; for example his putative [a] and [o] spectra are almost identical except for irrelevant overall amplitudes. Jordan, in fact, never claimed that chimpanzees produced human vowels, stating that their "sounds were not identical with those made by humans, but only resembled them." Although these deficiencies were noted seven years ago¹³, Marshall neglects this.

Marshall's comment concerning mynah birds misses the point of the paper he references¹⁴. Mynah birds mimic speech by producing energy peaks close to the formant frequencies, which human listeners are disposed to 'hear' as speech. Controlled psychoacoustic experiments with similar, computer-generated signals showed that they were as readily perceived as 'science-fiction noises' when listeners were not told that they would be hearing speech signals¹⁵.

Marshall implies that our work is irrelevant because the brain, which we supposedly ignore, is the biological basis of human speech. We have, however, consistently stated that the brain mechanisms necessary for human speech evolved in concert with the anatomy. The human SVT is relatively maladapted for swallowing and breathing^{4,16} but is able to produce the full range of human speech sounds, which would be impossible without specialized brain mechanisms.

The discovery of new fossil material, such as the Kebara hyoid by Arensburg *et al.*¹⁷ on which Marshall was commenting, is cause for excitement, but the authors' conclusions about Neanderthal speech may be premature. Nowhere do they show (except by dubious embryonic associations) how an unattached hyoid could be used to reconstruct the entire vocal tract. The hyoid is a free floating bone, suspended by muscles and ligaments, so knowledge of its exact position, and that of the larynx and the shape of the tongue, cannot easily be achieved¹⁸. Their

assertion that the Kebara hyoid resembles in some measures some modern humans, does not mean that its position in the neck was also similar. As we do not know what the hyoids of other fossil hominids looked like, it is possible that hyoid morphology was similar as far back as early members of *Homo*, if not earlier. If so, then the hyoid would be an irrelevant indicator of vocal tract evolution. Without other fossil hyoids to compare Kebara with, conclusions are meaningless.

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MARSHALL REPLIES—I do not believe that ablation of the tongue (body or blade) is a good idea (unless there is a compelling medical reason); and I agree with Lieberman and his colleagues that abnormality of the human vocal tract will reduce the intelligibility of speech. What I find startling is the 'plasticity' of the motor programs that can produce a 'fair approximation' to normal speech through a variety of articulatory manoeuvres over structurally-distinct anatomies. Ventriloquism and the ability to talk with a pipe in one's mouth are well-known examples.

More pertinent to the current argument is the study by Wheeler *et al.*¹⁹ whereby ten patients with oral cancer had 10–90% of the tongue ablated. After surgery, the intelligibility of the patients dropped by only 9–18%. In an earlier paper, Massengill *et al.*²⁰ reported a case who had 95% of his tongue removed but was "still able to communicate fairly well". The effects of human-vocal-tract abnormalities are addressed by Huskie²¹ in a recent book about cleft-palate speech. Huskie writes: "Some speakers are capable of surmounting seemingly major anomalies of the vocal tract to produce acceptable speech, while other speakers, with minimal structural defects present with marked dysfunction and atypical speech which appears to be out of proportion to the structural problem observed." I doubt that anyone with clinical experience would dispute this.

With regards to mynahs and chimpan-

zees, these creatures can produce acoustic output that is a sufficiently good approximation to human speech that a few sounds or 'words' can be interpreted as speech. They do so using mechanisms that are variously different from our own. Nobody claims that their vocal output is in all respects indistinguishable from our own, or that their vocalizations encode the phonological features that underlie human speech capacity. The point is simply that acoustic waves that resemble human speech can be produced by a wide range of very different mechanisms.

As to the Neanderthals, I am inclined to agree with Lieberman that they probably had some limited speech and language capacities. Perhaps they spoke Kabardian? But the evidence is as limited as their purported speech. Concerning the evidence from fossils, Du Brui²² has claimed that Lieberman's reconstructions imply a creature that could not open its mouth, let alone talk, and concludes that "the structure of the Neanderthal skull has been seriously misinterpreted". Lieberman and colleagues seem remarkably sure about the cerebral morphology and cognitive capacities of a species that has been dead and gone for a very long time.

Returning to the matter of the hyoid, I quote Luchsinger and Arnold²³: "If the hyoid bone has to be removed for surgical reasons, its absence is not noticed in any manner." Unfortunately, no reference is given for this remarkable assertion. Could some kindly surgeon (and speech scientist) please tell me if it is true?

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Seeing red in Queensland

SIR—Archaeologists studying rock paintings have commented on differences in the colours of paint used through time^{1,2}, with an apparent dominance in early paintings of reds over yellows. Study of rock paintings in north-western Queensland, Australia, such as the one shown here, has revealed that the art is undergoing a change on the rock face — goethite (αFeOOH), a yellow to brown mineral, is being converted to deeper colours in this Queensland rock art such as those found in low-temperature, humid, deep caves. Are some of the yellow-brown, brown and favourable conditions such as those found in low-temperature, humid, deep caves.



haematite ($\alpha\text{Fe}_2\text{O}_3$). We have also discovered rounded, goethite-dominated ochre fragments which, where exposed to extremes of weather, are being converted to haematite. Thus, under suitable conditions, the reaction: $2\alpha\text{FeOOH} \rightarrow \alpha\text{Fe}_2\text{O}_3 + \text{H}_2\text{O}$ is sufficiently rapid to influence the colouring of rock paintings.

The reaction has been studied to evaluate the relative stabilities of haematite and goethite. It has been shown that low relative humidity³, elevated temperature ($>40^\circ\text{C}$; ref. 4) and fine grain size ($<1\ \mu\text{m}$; ref. 5) all promote goethite instability. Climatic data⁵ and our grain-size measurements are compatible with the

abundance of haematite in older paintings and haematite forming from goethite in ochre fragments.

Rosenfeld¹ has drawn attention to the dominance of red in early paintings, but our studies suggest that this is not necessarily an indication that the paintings were monochrome red when applied. Perhaps yellow paint survives only in certain environmentally favourable conditions such as those found in low-temperature, humid, deep caves.

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Is cold fusion hot?

SIR—Observations of 2.5-MeV neutrons during the electrolysis of D_2O with palladium or titanium cathodes¹ have inspired non-standard models of $\text{d}+\text{d} \rightarrow {}^3\text{He}+\text{n}$ fusion. We suggested a less iconoclastic model², 'microscopically hot' fusion, in which deuterons are accelerated in local electric fields before fusing. In this case, $\text{d}+\text{t} \rightarrow {}^4\text{He}+\text{n}$ fusion will be favoured over $\text{d}-\text{d}$ fusion and yields a 14-MeV neutron more easily detected above background. Here we point out that experiments with tritium, judiciously pursued with proper regard for safety, may not only provide a decisive test of cold versus hot fusion but might also distinguish between different conditions of hot fusion.

As early as 1986, Derjaguin's group reported 2.5-MeV neutrons from sharply struck LiD crystals³. These neutrons were presumed to come from the fusion of deuterons heated in micro-pockets of plasma or accelerated to keV energies by electric fields. Such fields are associated with charged surfaces or isolated fragments induced by cracking. Deuteriding of a Pd or Ti cathode during electrolysis causes it to swell and distort and sometimes become brittle; hence we suggested

(ref. 2) that the resulting fusion neutrons might have origins similar to those from mechanically shocked LiD. Bursts of neutrons, and neutron production by other ways of shocking deuterated Ti or Pd were predicted² and have now been found^{4,5}. One objection to this model has been that, unlike LiD, deuterated Pd and Ti are not insulators, so that conduction electrons might be expected to quench any build-up of charge. But the Derjaguin group now reports generation of neutrons during the impact fracture of deuterated transition metals, including Ti (ref. 6). Also, model calculations suggest that a crack propagating with only $\sim 10\%$ of the speed of sound in Ti will outrun the electron current seeking to balance the charge⁷.

To obtain further predictions, we note that there are two sub-types of microscopically hot fusion: (1) heating (characterized by a high temperature) as would occur in a micro-pocket of plasma, and (2) acceleration (characterized by single-particle energies) as would occur across a crack. In case 1 the motion of both d and t nuclei is important; the cross-sections are calculated by²

$$\sigma_{a \rightarrow b} = \frac{S}{E_{\text{c.m.}}} \exp(-31.3\sqrt{\mu/E_{\text{c.m.}}}) \quad (1)$$

where S is the astrophysical S function (for $E_{c.m.} \leq 10$ keV, $S \approx S_0 = 54$ and 1.15×10^4 keV barn for $d+d \rightarrow {}^3\text{He}+n$ and $d+t \rightarrow {}^4\text{He}+n$, respectively), $\mu = m_a m_b / (m_a + m_b)$ is the reduced mass of nuclei a and b ($m_d = 2$ and $m_t = 3$ AMU) and $E_{c.m.}$ is the relative energy (in keV) having some thermal distribution. We thus have $\sigma_{d \rightarrow t} / \sigma_{d \rightarrow d} = 213 \exp(-2.99/\sqrt{E_{c.m.}})$, which is 11 at 1 keV and 83 at 10 keV.

In case 2 it is reasonable to expect only one of the reacting pair of nuclei to be accelerated and for the other to be essentially stationary on the other side of the crack. The electric potential is independent of the mass, so E_{lab} will be the same for accelerated d or t . But the d - t cross-section will then be different depending on whether d or t is accelerated, as can be seen by rewriting equation (1) as

$$\sigma_{a \rightarrow b} = \frac{m_a}{\mu} \frac{S}{E_{lab}} \exp(-31.3\sqrt{m_a/E_{lab}}) \quad (2)$$

where a is the accelerated particle and b is the stationary particle. Note that this formula is independent of m_b except for the weak dependence in μ . Equation (2) has the interesting consequence that $\sigma_{d \rightarrow t} / \sigma_{d \rightarrow d}$ is a constant equal to 178, independent of E_{lab} . (This ratio is in fact not constant if electron shielding is taken into account, but shielding is important only at $E_{lab} < 1$ keV, where the cross-section is too small to account for the observed neutron bursts; likewise at energies greater than 10 keV the S function varies, but such energies are unlikely to be produced by cracks.) Furthermore, $\sigma_{t \rightarrow d} / \sigma_{d \rightarrow t} \approx 1.5 \exp(-9.95/\sqrt{E_{lab}}) \ll 1$ at $E_{lab} \approx 1$ keV, so only the lighter particle is usefully accelerated, and hence the ratio of neutron yields is in effect still approximately constant.

Production of 14-MeV neutrons at a rate greater than that of 2.5-MeV neutrons in a 50:50 d - t mixture would prove that the fusion is really hot, but can hot fusion possibilities 1 and 2 be distinguished? In case 2 only accelerated deuterons react, so the total rate is reduced by a factor of two, but this factor is probably not helpful since we do not know the absolute rate to begin with. Fortunately, there is another possibility for discrimination, which comes from the ratio of 14- to 2.5-MeV neutrons. For case 1 this ratio depends on $E_{c.m.}$, and would reach and exceed the constant value applicable to case 2 only at energies higher than expected in a plasma. Also, in case 1 it is reasonable to

expect $E_{c.m.}$ and hence the neutron ratio to be different in different materials (such as Ti and Pd), while in case 2 the ratio should be sensibly independent of the material although the absolute rates might differ.

For either type of microscopically hot fusion, the probability of producing fusion conditions depends very much on the type of alloy, how it is treated, and the attention to detail of the experimentalist. This may account for the many null results, often at laboratories well versed in neutron detection⁸, and the frequent rumours of a

statistically significant result failing to be repeatable. Identification of the fusion mechanism should enable choices yielding more reproducible results.

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Antidote to taxonomic instability

SIR—The current instability of biological nomenclature is increasingly irksome¹⁻³, and the antics of the lunatic fringe of taxonomy bring the whole discipline into disrepute⁴. Legitimate name changes include the exposure of past errors in the recognition of species, not the exhumation of overlooked homonyms⁵.

But revisions of classification may also trigger changes in nomenclature. The aim of most taxonomists is to arrange species into monophyletic taxa — natural groups whose members are supposed to share a single common ancestry. Paraphyletic taxa (that is, incomplete monophyletic taxa) are avoided on principle, but in practice one is always confronted by 'residual paraphyletic groups'⁶. These may be created when the rank of a subset of species within a genus is raised to the generic level: the remainder, named under the original genus, then constitutes a paraphyletic group which cannot be defined by a unique set of characters. It has been common practice in the past to conceal the prevalence of residual paraphyletic groups.

Many name changes result from raising the ranks of taxa in order to avoid paraphyly. The alternative option of demoting the excluded taxa to the next subordinate rank, to avoid the paraphyly resulting from their exclusion from the related monophyletic taxon of the same rank, is rarely considered. For example, new evidence suggested the insect family Empididae was paraphyletic, because of the exclusion of the Dolichopodidae. One solution was to raise the subfamilies in the Empididae to familial rank, the rank of the Dolichopodidae⁷. This had the effect of creating several new family names and restricting the definition of the Empididae. But if the rank of the Dolichopodidae had been reduced to that of a subfamily, the Dolichopodinae, the only consequential nomenclatural change would have been this one letter ('d' to 'n').

In considering the change in nomenclature, we need to distinguish between those subordinate taxa that are raised to generic rank without any reference to scientific

arguments and those that are raised to avoid paraphyletic taxa. The former are done *ex cathedra* or else for the trivial reason that more detailed study has revealed subordinate groupings within a taxon.

The problem with the nomenclature of paraphyletic genera, in particular, derives from the requirement that the generic name is used as part of the formal species binomen — despite the knowledge that we are likely to be stuck with residual paraphyletic groups indefinitely. I propose that we should identify residual paraphyletic genera by calling them 'paragenera'. Furthermore, we should allow the first word of a species binomial to be either the name of the genus or the paragenus to which the species belongs. The rule of priority should apply, regardless of any subsequent resolution of the paraphyly of a paragenus by recognition of constituent monophyletic 'genera'. Likewise if the paraphyly of a paragenus is resolved by adding to it the excluded genera, thus demoting the latter to the subgeneric rank, the priority of their 'generic' names should continue to be recognized. In this case a subgeneric name would form the first word of the binomen.

This proposal would mean that the generic name would normally be the first word of the binomen, as is the current rule, but would in a few cases be a paragenus or a subgenus. The increase in stability of nomenclature that would result from this radical modification of the accepted rule would far outweigh the disadvantages. It should not be rejected out of hand by those unprepared to offer an alternative to the current rampant instability.

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Cognitive network news

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Neural Connections, Mental Computation. Edited by Lynn Nadel, Lynn A. Cooper, Peter Culicover and R. Michael Harnish. MIT Press: 1989. Pp. 355. \$39.95, £31.50

Neural Computing Architectures: The Design of Brain-Like Machines. Edited by Igor Aleksander. Chapman and Hall: 1989. Pp. 401. £35. Distributed by MIT Press in the United States: \$45.

Computer Simulation in Brain Science. Edited by Rodney M. J. Cotterill. Cambridge University Press: 1988. Pp. 566. £50, \$65.

IN THE first golden age of neural networks, around 1960, Frank Rosenblatt, originally a psychologist, proposed the perceptron as a simple network model for brain function. H. D. Block, a physics-orientated mathematician, proved theorems on perceptron properties while Bernard Widrow, an electrical engineer, studied a related model, ADALINE (adaptive linear neuron) and created the now famous Widrow-Hoff learning algorithm for training the network to solve useful pattern-discrimination problems. Psychology, physics and electrical engineering, rather than neurobiology, were the birthplace of the neural-network model.

But it was experimental neurobiology, and not neural networks, that flourished for the next two decades. Abandoned electronic perceptrons built from vacuum tubes and servo potentiometers rusted in a barn outside Ithaca, New York, while stimulators in London and Oslo pulsed hippocampal pathways demonstrating biological synaptic learning algorithms in the brain. Network pioneers, including Widrow, continued their work, but it was only after the farm was sold and the perceptron relay racks thrown out for good that the second age of expanding neural-network, or 'connectionist', research began. Not everyone agrees that neural-network research is in its second golden age, but the conference proceedings reviewed here demonstrate that psychology, physics and electrical (now largely computer) engineering are important disciplines still providing a driving force behind much of the renaissance. The books differ markedly in emphasis with respect to these disciplines and also in the degree to which faithful representation of the nervous system is seen as the goal.

Neural Connections, Mental Computation arose from a meeting (dedicated to D.O. Hebb, originator of the Hebb learning rule) of psychologists, cognitive scientists and neuroscientists, at the University of Arizona in 1986. The aims of the meeting were to answer specific questions concerning contemporary connectionist modelling of psychological phenomena and brain function. Part 1 addresses the question of whether connectionist networks can adequately describe psychological phenomena. Here, cognitive sci-

entists and connectionist modellers join forces to attempt to explain how information is represented in networks — for example, how symbolic structures might be interpreted in the framework of distri-

structured 'features' as inputs to the network, but ignore how the structure of the network itself might reflect the physics of the problem to be solved, preferring instead to 'learn' this architecture. Given our present theoretical understanding of neural-network learning algorithms and the extraordinarily difficult problem of teaching unstructured networks to generalize from limited training data (covered in more detail in the other volumes reviewed here), this learn-it-all approach is disappointingly naive. Thus the issues Shepherd raises are likely to be at the forefront of future research.

Part 2 deals with the constraints that biological neural circuits impose on the connectionist theorist who wants faithfully to adhere to what is known of brain

architecture. Most of the contributions here are limited to accounts of models inspired by hippocampal anatomy and synaptic physiology, and reflect the research interests of the conference organizers. Despite this emphasis on one brain subsystem, the goal of describing the computational functions of brain areas by relating their architecture to somewhat simplified connectionist networks containing basic architectural features and learning rules of the biological system is well represented. McNaughton, for example, tries admirably to bridge the gap between single-cell physiology, network architecture of hippocampal fields and the hypothesized computational function of the hippocampus as contributing to a cognitive map of the physical world through 'place' cells.

This well-organized, solid contribution of material is relevant to the interpretation of connectionist models in cognitive science and contains several examples of attempts to understand complex cortical circuitry from the perspective of simplified network models.

The book contains contributions from a relatively small meeting, and the quality of nearly all of the papers is high; as such, it successfully contributes to the rising interest in connectionist models in psychology and neuroscience.

Neural Computing Architectures: The Design of Brain-Like Machines is intended both as a comparison with and as a response to the two-volume classic *Parallel Distributed Processing*, edited by D.E. Rumelhart and J.L. McClelland and published by MIT Press in 1986. As well as to provide a "guide to architectural issues that arise in neural computing", the editor aims to present the view of European re-



Fast thinking — Solomon Stone, 'the greatest mental calculator in the United States', 1890*.

buted representations across the simple processing units and connection weights that comprise the network. These are important issues in relating psychological phenomena to the structure and function of the brain, and are generally ignored in other books on neural networks.

A contribution of particular insight is by Roger Shepherd, who summarizes his theoretical ideas concerning how physical laws governing objects in the real world must necessarily be expressed, through evolution, in the detailed architecture of brain-processing areas. Most applications of connectionist networks assume a set of

searchers in the way that the PDP volumes represent the work of those in the United States. Indeed, a 60-page summary of the PDP volumes constitutes the final section of the book to aid direct comparison. In addition to its geographical specialization, the commissioned papers (16 of them) from European researchers have a decided emphasis on electrical engineering and physics as applied to artificial neural networks, and little is to be found that relates to the functioning of the nervous system.

A few of the main players in the history of European neural-network research are represented. Caianiello, one of the early pioneers, summarizes his theoretical work, which he has carried out for 30 years. Similarly, Kohonen discusses the application of his self-organizing feature maps (an inspired network model that is yet to be fully explored) to speech-recognition problems. But given the strong tendency of PDP models to relate to psychological and brain function, it is unfortunate that European contributors who are attempting to relate network models more closely to real brain function are not represented — for example, von der Malsburg's models of the visual cortex; Reichardt's computational models of the fly's visual system; and Willshaw's neurally inspired learning algorithms.

To my mind, the book seems misguided for another reason: the work represented in the PDP volumes, although a strong influence on neural-network research, by no means defines the field in the United States. Contrary to its stated purpose, the new book is a loosely organized, sparsely sampled assortment of European neural-network researchers and seems to reflect the particular research interests of the editor. Part II ("The Logical Perspective"), constituting one-third of the research material in the volume, presents a collection of work primarily related to practical pattern-recognition systems based on boolean function units (the editor's system WISARD being the best example). This may be useful to those interested in the implementation of binary neural networks in digital computer hardware, but those interested in understanding the many important historical and contemporary contributions made by Europeans to neural-network research would be better off studying the well-organized and thoughtfully edited collection of reprints in *Neurocomputing: Foundations of Research* (eds J.A. Anderson and E. Rosenfeld, MIT Press, 1989).

They might also, perhaps, look at the third book reviewed here, *Computer Simulation in Brain Science*, whose 35 chapters are transcribed from talks presented at a conference held in Copenhagen in August 1986. The book's title aptly describes the theme around which the meeting was organized: how the com-

puter has been used as a tool in the study of the brain. Here there is a broad international representation of research. Focused on the application of computer technology as opposed to a scientific question, the conference displayed a diverse collection of research topics. Almost all the papers bear some relationship to neurobiology, but a few deal with very abstract, non-biological connectionist networks, cellular automata and deterministic chaos. The "lack of empirical data on modern large-scale wars" even drew a battle-management specialist to Copenhagen to compare notes with brain modellers on distributed adaptive control strategies.

The modelling work runs the full scale from the microscopic to the macroscopic: simulations and modelling of enzyme dynamics, dendritic branches, single-neuron electrophysiology, cortical network models and beyond. As transcripts of the talks rather than contributed research papers, the chapters frequently have a casual style and, in most cases, provide only a bare-bones summary of the authors' research. The full scale of quality is also evident. But the papers do provide useful bibliographies of the original publications, and the book will be beneficial to those interested in keeping up with the growing international community of those who study brain function by reference to neural models and computer simulations.

None of the books reviewed here is for the newcomer to neural computation. Those eager to understand current directions in research would be advised to avoid the sensory overload likely to result from parallel processing of such a bewildering array of theories and models, and first to accomplish a slow, serial iteration through *Neurocomputing: Foundations of Research*. A textbook of neural-network concepts and ideas remains to be written. □

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*The picture was reproduced from *The Science of Mind* by K. Klivington. Published by MIT Press the book is available in the United States and to be published in Britain, January 1990. Prepublication price \$29.95 (until January 1990) £26.95 (until April 1990); price thereafter \$39.95, £35.95.

• *Methods in Neuronal Modeling: From Synapses to Networks*, edited by C. Koch and I. Segev, is a volume of papers collected from a course entitled 'Methods in Computational Neuroscience'. The volume discusses techniques for numerical modelling of the nervous system and is published by MIT Press, price \$45, £40.50.

• Another recent publication, *The Computing Neuron*, edited by R. Durbin *et al.* includes a selection of work presented at a meeting on 'The Neuron as a Computational Unit' and is published by Addison-Wesley, price £24.95.

Life's formula

Peter Keightley

Mathematical Evolutionary Theory. Edited by Marcus Feldman. Princeton University Press: 1989. Pp.341. Hbk \$60, £37.50; pbk \$19.95, £12.50.

AT ONE extreme, theoretical models of evolutionary processes relate to molecular evolution of the genetic material itself; at the other, they are to do with the evolution of culturally transmitted characters. *Mathematical Evolutionary Theory* is a collection of articles dedicated to Samuel Karlin, whose interests span the full range of the subject. The contributions have been written by leading workers who have been closely associated with Karlin or influenced by his research. Each author has produced an article based on his own research, with varying attempts to introduce the background to the subject: some take the form of an original research paper, while others are very close to straight review articles.

The book is divided into two sections, with an introduction by the editor containing short but helpful summaries of each paper. The first section ("Stochastic and Deterministic Genetic Theory") contains contributions of a general nature that examine the theoretical consequences of phenomena such as genetic drift, population subdivision, linkage disequilibrium and migration as they affect genetic variation and rates of genetic change. The second ("Behavior, Ecology, and Evolutionary Genetics") deals with more 'applied' problems, and includes accounts of such topics as the evolution of culturally transmitted characters, kin-selection, the theory of evolutionarily stable strategies and molecular evolution. The diversity of the papers, even within each section, is such that any one of them is likely to be of direct interest to only a few readers.

Among the contributions I found particularly stimulating is that by Ewens, which addresses the problem of the effect of population subdivision with local extinction and recolonization of demes on effective population size and the genetic variation that can be maintained at equilibrium. Models of this nature have long attracted the attention of population geneticists, and are clearly of biological interest. Ewens shows that such a subdivided population can maintain more genetic variation than a non-subdivided one. Weir and Cockerham present a complete analysis of disequilibrium at two loci and obtain maximum-likelihood estimates for disequilibrium coefficients. These somewhat formidable formulae are used to determine whether the disequilibrium observed in a sample is within the bounds of what might be expected if the

population were free from disturbing forces. The work therefore has practical implications.

In the second part of the book, Roughgarden presents a review of the evolution of marine life forms. To those such as myself who are unfamiliar with the subject, this article raises the interesting question of an apparent dichotomy in marine life-cycle types. For adult organisms which have a purely benthic (bottom-dwelling) life-style, the larvae are almost invariably either purely benthic or purely pelagic. Using a mathematical model, Roughgarden attempts to interpret the evolutionary aspects of the dichotomy.

Also in the 'applied' section, Kaplan and Hudson investigate a model for the evolution of families of dispersed, highly repeated DNA sequences in mammals. Such families probably originated by a mechanism involving reverse transcrip-

tion, and their spread may also be mediated by this mechanism. Under the assumption that at any time only one copy is 'active' in transposition, Kaplan and Hudson derive formulae for the expectation and variance of time to the most recent common ancestor of a pair of sequences. The results from the model are shown to compare favourably with results from an analysis of data from several mammalian species.

Mathematical Evolutionary Theory contains a summary of research at its leading edge. Many of the contributions contain difficult algebra, which will limit their appeal, but by picking and choosing, determined readers will find here a great deal of value. □

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small a mass of oil could cover such an immense area, he did not go on to ask the 'right' question: "How small is a molecule?" Tanford's explanation shows historical sensitivity and a refreshing absence of Whiggish historiography. A century later the enquiry was resumed by Lord Rayleigh, continued by Agnes Pockels and brought to a triumphant conclusion by Irving Langmuir. For the multitudes of chemists who have used the Langmuir trough, or otherwise dabbled in surface chemistry, the story is of very considerable interest.

But it does not end there. From chemistry we are transported to biochemistry and the recognition in the 1970s of the phospholipid bilayer as the universal structural framework of all biological membranes. Ben Franklin would have been well pleased.

This is a good history by a scientist, a lot better than many. But it displays some justification for the caveats of professional historians. There are numerous minor mistakes — Wilcke was not a professor (p. 32), and Davy was not "Humphrey", for instance. Nor, these days, would many historians be happy with the statements that Priestley was *the* discoverer of oxygen (p. 39), that Dalton equated "elements with atoms" (p. 58), or that Kelvin's later attitudes to the age of the Earth were "intransigent" (p. 131).

Tanford is least confident when dealing with matters away from science. He apparently does not realize that the "itinerant preacher from Ireland, the Reverend Mr Whitefield" was the world-travelled evangelist and co-founder of Methodism, George Whitefield (a good friend of Franklin). And he is hardly fair to "the

In review

Colin Russell

Ben Franklin Stilled the Waves: An Informal History of Pouring Oil on Water with Reflections on the Ups and Downs of Scientific Life in General. By Charles Tanford. *Duke University Press: 1989.* Pp. 228. \$33.50, £26.80.

UPWARDLY mobile historians of science have occasionally displayed a strident contempt for essays in their subject not emanating from their own community or from other historians, reserving their best invective for histories written by scientists. Such voices are not necessarily young, are fortunately unrepresentative but may nevertheless be loud.

To an outsider these protests suggest merely the narrow parochialism and arrogant pedantry of an insecure, embattled and inward-looking community. From within, however, they articulate a deep concern that important and distinctive insights may be lost, together with many things that historians have had painfully to learn: the inverse ratio between historical objectivity and proximity to one's subject, the demonstrable importance of social and cultural context, as well as innumerable tricks of the trade for extracting evidence from neglected, unlikely and diverse sources. Yet it has always seemed to me extraordinarily naive to condemn a whole genre of writing just because it is different. To do so ignores two points: one is that there must surely be more than one kind of historical writing (and certainly more than one kind of audience for it). The other is that direct experience of the scientific enterprise gives insights and depths of understanding that are just as valid as the nuances

beloved by the historian.

All of which is to explain why I applaud this brave attempt by a distinguished expert in the field of biological molecules to recount the development of our understanding of monomolecular and bimolecular layers. It is a truly important chapter in the history of surface chemistry and biochemistry, recounted in a lively, even rumbustious, way. There are plenty of personal anecdotes, most of them relevant and nearly all entertaining.

Serious study of the subject began when Benjamin Franklin showed how a drop of oil spreads over an aqueous surface, in a pond on Clapham Common, on Derwentwater, or calming a storm at sea. Although he recognized the curious fact that so

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A friend of Franklin — Reverend Mr Whitefield preaching at Leeds, 1749.

Reverend Mr Farish", whose curiosity led to Franklin's experiment being recorded in the *Philosophical Transactions*. James Farish is the fall guy of Tanford's book, simply because he failed to draw the necessary conclusions from Franklin's experiment. He is "unimportant", "foolish", "naive" and perpetrator of "the Farish syndrome: failure to learn". Yet "poor Mr Farish" was a very learned man, something of a polymath, whose chief fault was reported as a reluctance to go

into print. He married into the famous Gilpin family and appears in the *Memoirs of Richard Gilpin*. His son William was Professor of Chemistry at Cambridge. He was not a good choice as Simplicio.

Enough! Tanford has written a fine account of an intriguing aspect of chemical and biochemical history, one from which even historians can learn a great deal. □

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Universal theory

Iwan Williams

The Formation and Evolution of Planetary Systems. Edited by H. A. Weaver and L. Danly. Cambridge University Press: 1989. Pp.353. £30, \$44.50.

The Origin of the Solar System: The Capture Theory. By John R. Dormand and Michael M. Woolfson. Ellis Horwood: 1989. Pp.230. £29.95. Distributed by John Wiley in the United States, \$58.60.

THE origin of the Earth and other planets of the Solar System has been a topic of discussion among intelligent human beings ever since they became intelligent. After Copernicus had established the true dynamical nature of the system, theories became more scientific and today we are essentially still in debate over the correctness of ideas formulated 200 years ago.

We have made some progress, of course, and we have very much more data available now, as a consequence of both space exploration and improved ground-based observation. Indeed, in the 1960s and 1970s there was an explosion in the number of workers in the field, and at one stage it was a standard joke that Al Cameron would produce a new theory for every meeting. The 1980s have been much more a time for consolidation. There have been relatively few books published on the topic over the past few years, so it comes as a surprise to find two of them appearing almost simultaneously.

The Formation and Evolution of Planetary Systems is the report of a meeting on the topic and more or less presents the current consensus view. It is an edited work, with various contributors looking at different aspects of the problem, and is not therefore a comprehensive account of the formation of planets. By contrast, *The Origin of the Solar System* by Dormand and Woolfson presents the authors' own, more controversial, view; here we do have the (or rather a) complete story starting with interstellar material and finishing with a planetary system. As might be expected, the books are almost mutually exclusive, with no reference to Dormand and Woolfson's work in the Weaver and

Danly volume, while only the work of Cameron, of all the contributors in that book, is mentioned by Dormand and Woolfson.

It is almost self-evident, and has been so for several centuries, that there are fundamentally only two ways of making planets — either you break up something

within a gaseous envelope or disk surrounding the Sun. Close to the Sun the grains are composed of the non-volatile materials only, and the final aggregated planets are similar in composition, and small, as such material is not abundant. Further out water exists as ice, which results in much more material condensing and bigger embryo planets. Such embryos are able also to capture the free gas and so the end-product is a large gaseous planet.

Once a theory becomes fashionable, many people start to work on it and it then becomes possible to start an investigation at an arbitrary point in the story and possibly not continue to the final emergence of a finished planet. This is the situation with the Weaver and Danly volume, where individual chapters deal with isolated bits of the overall problem. This is fine for current workers in the field, but will not be

Michael Holford Photographs

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Model behaviour — the relative motions of the planets within the Solar System are shown on an orrery by means of clockwork mechanics. Orreries became fashionable instruments to own during the eighteenth century when they were of practical and ornamental use. These clockwork Solar Systems were named after Charles Boyle, Earl of Orrery, for whom one was made in 1713.

This particular miniature was made by Troughton in the early part of the eighteenth century, and is at the Science Museum, London.

big or you build them up from small bits. Dormand and Woolfson go along the first route and envisage the capture of material from a passing proto-star by the young Sun. This material fragments under its own gravity to form spherical proto-planets on orbits not dissimilar to those of the present planets. These proto-planets would initially be gaseous but self-gravity causes any solid grains to settle to the centre. Those nearest to the Sun lose most of their outer gaseous layers while the others retain most of the gas. The book gives a very readable account of all the computer modelling that has been carried out to verify the model, which has fewer flaws than is claimed by opponents who have not studied it.

Most planetary scientists have gone along the alternative road, however, and primarily consider formation of planets through a build up of small objects, mainly by the aggregation of condensed grains

particularly helpful for astronomers who are specialists in other areas (of course, it was never intended that the volume should have that purpose).

These two books are very different and have different aims. The conference proceedings take a look at the current state of research as perceived by conventional wisdom and highlights potential problems which should be tackled, by both theorists and observers. Within this context, it will be useful to new workers in the field and to the older generation who have perhaps drifted into other areas. Dormand and Woolfson present a single unique theory for the formation of planets. Their book deserves to be read by the planetary science community; although I have my doubts, they may just be correct. □

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Evidence for stratospheric nitric acid condensation from balloon and rocket measurements in the Arctic

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Strong evidence for nitric acid condensation at altitudes of about 18–23 km is provided by balloon- and rocket-borne measurements made near Kiruna, northern Sweden, in January 1989. Nitric acid condensation is believed to be a necessary step in the chemical pre-conditioning required for chlorine-catalysed ozone destruction.

STRATOSPHERIC nitric acid condensation has been proposed^{1,2} to occur in the winter polar stratosphere, probably leading to solid aerosol particles possessing a composition close to that of nitric acid trihydrate, $\text{HNO}_3 \cdot 3\text{H}_2\text{O}$ (NAT). It was also suggested that NAT aerosols set the scene for chlorine-catalysed ozone destruction. In particular, nitric acid aerosols may serve as catalysts for surface-catalysed reactions converting reactive nitrogen gases to HNO_3 and chlorine reservoir gases (mainly HCl and ClONO_2) to photolytically active chlorine-containing gases, from which chlorine atoms can rapidly be liberated after the end of the polar night. The passivation of reactive nitrogen through conversion to HNO_3 is a necessary condition for chlorine activation because NO_2 induces rapid repassivation of active chlorine. Photolysis of gaseous HNO_3 re-forms NO_2 and therefore the concentration of HNO_3 in the gas phase is of critical importance. After NAT-aerosol evaporation, in the presence of sunlight, HNO_3 photolysis re-forms NO_2 within 1–2 weeks. If, however, nitric acid becomes incorporated into relatively large (several μm) aerosols, which undergo significant sedimentation, HNO_3 may be removed from a cold layer so that rapid re-formation of NO_2 by HNO_3 photolysis cannot take place. Chlorine-catalysed ozone destruction should be more efficient under these conditions.

The idea of stratospheric nitric acid condensation was further developed through model calculations^{3–8}, laboratory measurements^{9–19}, and atmospheric measurements^{20–25}. Various indirect arguments indicate strongly that nitric acid condensation does, in fact, occur in the cold stratosphere. There has been, however, no direct proof and the altitude variation of the threshold temperature $T_c(\text{NAT})$ for stratospheric nitric acid condensation is still uncertain. The indirect arguments include: (1) laboratory measurements¹² of the equilibrium saturation HNO_3 and H_2O vapour pressures for macroscopic surfaces of $\text{HNO}_3/\text{H}_2\text{O}$ mixtures at low stratospheric temperatures. These allowed better model calculations^{11,12,20,21} of $T_c(\text{NAT})$ and therefore of the temporal and spatial extent of nitric acid aerosols; (2) balloon measurements²⁰ of the stratospheric altitude distribution of gaseous nitric acid in the Arctic winter polar vortex under conditions of temperatures above T_c , which have set upper limits for T_c of 195 K (18 km) and 193 K (23 km); (3) aircraft measurements²¹ of total NO_y in the Antarctic winter polar vortex which revealed NO_y to be contained in stratospheric aerosols under low-temperature conditions; (4) aircraft-borne sampling experiments^{22,23} of stratospheric aerosols followed by laboratory analysis which disclosed nitrate being contained in the aerosols.

Here we report three *in situ* measurements of the detailed

altitude distribution of gaseous nitric acid in the Arctic winter polar vortex under conditions of extremely low temperatures. Two measurements show that there is strong local depletion around 20 km which we conclude is a manifestation of local nitric acid condensation inducing the formation of nitric acid aerosols. Below the HNO_3 depletion layer, HNO_3 was markedly enhanced which was probably due to vertical redistribution of HNO_3 by aerosol sedimentation. We discuss the implications for polar stratospheric nitric acid aerosols and their potential effect on trace-gas concentrations, primarily ozone.

Measurements

We measured the stratospheric gaseous nitric acid using balloon- and rocket-borne ACIMS (active chemical ionization mass spectrometry) instruments. We have developed ACIMS as a method for the measurement of stratospheric and free tropospheric trace gases and have thoroughly described it previously^{20,26–30}. ACIMS relies on the attachment of trace-gas molecules to ions leading to characteristic product ions. By mass spectrometric analysis of product and precursor ions the concentration of the trace-gas species can be determined with high sensitivity and time resolution. The latter offers a high spatial, specifically

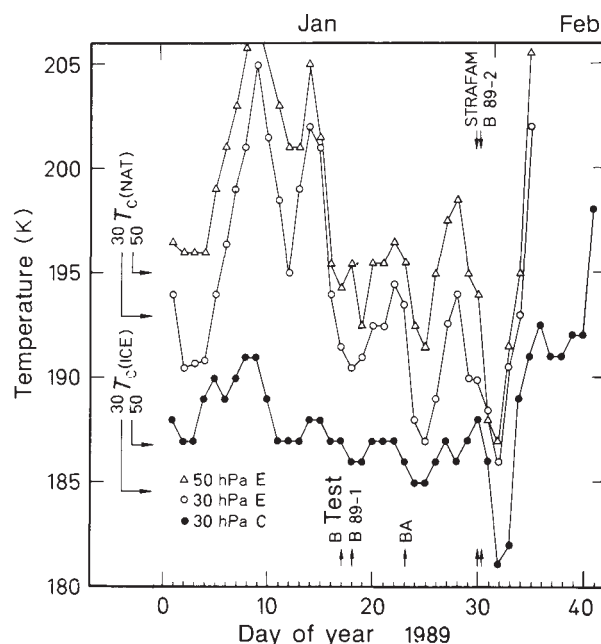


FIG. 1 Time evolution of stratospheric temperatures (January and February 1989) at the 30-hPa and 50-hPa pressure levels (corresponding to ~22 and 19 km) above Esrange (E) and at the centre (C) of the Arctic cold region. The data were obtained from stratospheric weather maps provided by the Free University of Berlin (K. Labitzke, personal communication). Balloon (B) and rocket STRAFAM flights are indicated by vertical arrows. Also given are model condensation temperatures for NAT and water ice (horizontal arrows) for the 30-hPa and 50-hPa pressure levels (see text).

altitude, resolution. For HNO_3 detection the reaction of $\text{CO}_3^-(\text{H}_2\text{O})_n$ with HNO_3 leading to $(\text{CO}_3\text{HNO}_3)^-(\text{H}_2\text{O})_{n-1}$ is used²⁹. The rate constant k is $1.4 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$ (ref. 31). (In our previous HNO_3 measurements²⁰ we assumed a rate constant of $2 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$ and therefore our previous data have to be corrected, yielding nitric acid concentrations that are higher by a factor of 1.43.) The uncertainty and precision of the measured HNO_3 concentration are $\pm 40\%$ and $\pm 20\%$, respectively. Another potential source of uncertainty may be partial evaporation of NAT aerosols in the flow tube of the ACIMS instrument, leading to an over-estimation of gaseous nitric acid. This perturbation should, however, occur only under conditions when most by far of the available nitric acid is contained in NAT aerosols (see below).

Our measurements (two balloon flights and one rocket flight) took place in January 1989 at Esrange (68°N , 21°E —northern Sweden—50 km east of Kiruna) within the framework of the international TECHNOPS campaign which also included a third scientific balloon flight to measure other³² stratospheric trace substances. The two balloon flights (B89-1 and B89-2) were carried out on 18 and 30 January 1989. B89-1 carried only our ACIMS instrument, but B89-2 carried, besides our ACIMS instrument, other instruments³² including ozonesondes, an optical aerosol counter and a condensation-nuclei detector. ACIMS measurements were made during ascent and parachute descent covering the altitude range between ~ 7 km and 27 km (B89-1) and 7 km and 31 km (B89-2).

The rocket-borne ACIMS instrument was launched at 09:30 LT on 30 January 1989 at Esrange. Here, ACIMS measurements were performed during parachute descent at altitudes between about ~ 65 km and 15 km, at a distance up to ~ 50 km north of Esrange. (A brief description of the rocket-borne ACIMS instrument along with first scientific results will be given elsewhere³⁷.)

We planned our ACIMS measurements to take place when the stratosphere above Esrange is extremely cold. From our previous measurements²⁰ and model estimates^{12,20} it seemed that $T_c(\text{NAT})$ must be $< 195 \text{ K}$, more likely $\sim 193 \text{ K}$, at 20 km. The lowest temperatures in the Arctic winter stratosphere usually occur at altitudes around 20 km. Figure 1 displays the time evolution of stratospheric temperatures during January and February 1989. Five pronounced minima occurred in the temperatures above Esrange (at the 30-hPa and 50-hPa pressure levels corresponding to ~ 22.3 and ~ 19.3 km). These are at least partly the result of rotation and deformation of the polar vortex. Well inside the vortex, temperatures undergo much less pronounced temperature fluctuations (see Fig. 1) than at Esrange, which is mostly located in the vicinity of the vortex boundary. Because these five minima, although less pronounced, are also observed at the core of the vortex, it seems that large-scale upwelling, leading to adiabatic cooling, must also have been involved. The balloon and rocket flights reported took place close to two of these minima with somewhat lower temperatures prevailing during the rocket flight and B89-2 on 30 January. On 30 January, the vortex was substantially displaced from the geographic North Pole towards the European sector covering large parts of Scandinavia and the northern European Soviet Union so that a region with particularly low temperatures developed mainly over Scandinavia. On that day the vortex boundary was located above southern Scandinavia (R. Jones, personal communication) and therefore B89-2 and the rocket should have encountered stratospheric air masses that are characteristic of the interior of the vortex. By contrast, on 18 January the vortex boundary was located only slightly south of Esrange.

Nitric acid profiles

The three altitude profiles of gaseous nitric acid concentrations measured by the balloon- and rocket-borne ACIMS instruments are shown in Fig. 2. The B89-1 profile (Fig. 2a) is relatively

smooth with a broad maximum around 18–25 km, where concentrations of $\sim 1.5\text{--}2.0 \times 10^{10} \text{ cm}^{-3}$ are reached. In terms of volume mixing ratio, the maximum (~ 22 p.p.b.v., parts per 10^9 by volume) is located around 25 km. Ascent and descent data are in good agreement even though, because of wind shear, they were not made in exactly the same air mass. The B89-1 profile is similar to previous nitric acid measurements made in the Arctic winter. Above 23 km the B89-1 values are mostly larger (up to a factor of about two) than the monthly (January) and zonally (68°N) averaged LIMS (Limb Scanning Infrared Monitor of the Stratosphere) profile of 1979 (experiment flown aboard the Nimbus 7 satellite), which is also shown in Fig. 2a. Below 23 km the two profiles are similar. The profile obtained from a balloon-borne ACIMS instrument launched on 1 February 1988 at Esrange (B88-2) (also given in Fig. 2a) is lower than the B89-1 profile by up to a factor of two. (The B88-2 data have been corrected upwards by a factor of 1.43 to account for the measured $k = 1.4 \times 10^{-9} \text{ cm}^3 \text{ s}^{-1}$.) The B89-1 total vertical column density of HNO_3 ($3 \times 10^{16} \text{ cm}^{-2}$) is comparable to previous values measured during the Arctic winter^{27–29} ($2\text{--}3 \times 10^{16} \text{ cm}^{-2}$). It is at least conceivable that the somewhat higher B89-1 data are the result of further nitric acid formation by aerosol surface-catalysed conversion of reactive nitrogen to HNO_3 during low-temperature events. Upon subsequent warming gaseous HNO_3 may have been released leading to an overall HNO_3 increase. The B89-2 profile (Fig. 2b) differs quite substantially from the B89-1 profile at altitudes between about 13 and 23 km whereas there is reasonably good agreement at other heights. Again ascent and descent data of B89-2 agree reasonably well with each other. Around 18–23 km there is a local minimum or a bite-out (with respect to B89-1) whereas around 13–16 km there is a local maximum or an excess (with respect to B89-1). The latter is more pronounced in the descent data where maximum concentrations of $\sim 4 \times 10^{10} \text{ cm}^{-3}$ (23 p.p.b.v.) are reached around 14 km. These are larger than the LIMS and B89-1 data at 14 km by a factor of about four. Figure 2c gives a composite picture of the balloon and rocket profiles. Interestingly the rocket profile also exhibits a bite-out around 17.5–23 km, similar to profile measured onboard B89-2 which flew on the same day. At most heights the bite-out in the rocket profile is more pronounced than that of B89-2, particularly at 19.6 km where the rocket value (upper limit) is extremely small.

Possible explanations for the local maximum around 14 km observed in B89-2 are horizontal transport of HNO_3 -rich air or gravitational sedimentation of HNO_3 -containing aerosols followed by evaporation around 14 km which liberates gaseous HNO_3 . The former explanation is not supported by ozone measurements made onboard B89-2, which do not show enhanced ozone around 14 km (ref. 32). The large HNO_3 concentration measured around 14 km is difficult to explain in terms of only horizontal transport because it substantially exceeds the typical HNO_3 concentrations predicted by models and observed by LIMS. Vertical redistribution involving sedimentation of relatively large HNO_3 -containing aerosols (NAT or water ice with dissolved HNO_3) seems to be more likely. For example, a spherical NAT aerosol particle with a radius of $5 \mu\text{m}$ has a sedimentation velocity, at ~ 18 km, of $\sim 1 \text{ km d}^{-1}$. So it would fall from 20 to 14 km within about one week. After having sedimented out of the layer with $T \leq T_c(\text{NAT})$ an aerosol particle evaporates rapidly. The nitric acid initially contained in the particle may therefore be liberated and deposited within a relatively thin layer located just below the cold layer.

The aerosol size and therefore its sedimentation velocity depend critically on the fraction of pre-existing $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ aerosols which serve as active nuclei for nitric acid condensation. If this fraction is small, particularly large NAT aerosols will be formed, which sediment more rapidly. For example, if that fraction is only 0.0001 the HNO_3 flux may be as large as several tens of p.p.b.v. per km per day³³. If temperatures become

sufficiently low to allow condensation of 1 p.p.m.v. water the fraction of active nuclei may be as large as 0.01 for the same HNO_3 flux³³. It is conceivable that on 30 January stratospheric air masses that had previously experienced sufficiently low temperatures to allow NAT or even water-ice aerosol formation were advected to northern Scandinavia, leading to relatively large aerosol particles. An inspection of Fig. 1 reveals that the

lowest 50-hPa temperatures in the vortex had occasionally even lower than $T_c(\text{ice})$ before 30 January.

The bite-out observed on 30 January between 18 and 23 km could be the result of (1) local HNO_3 condensation leading to NAT aerosols that were still present in that layer; (2) HNO_3 removal from that layer by prior HNO_3 incorporation into aerosols ($\text{HNO}_2/\text{H}_2\text{O}$ condensation or HNO_3 dissolution into

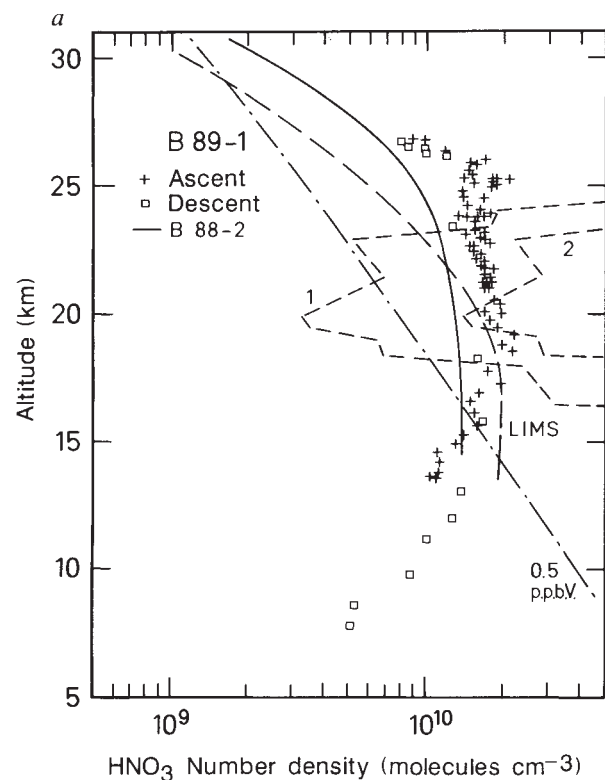
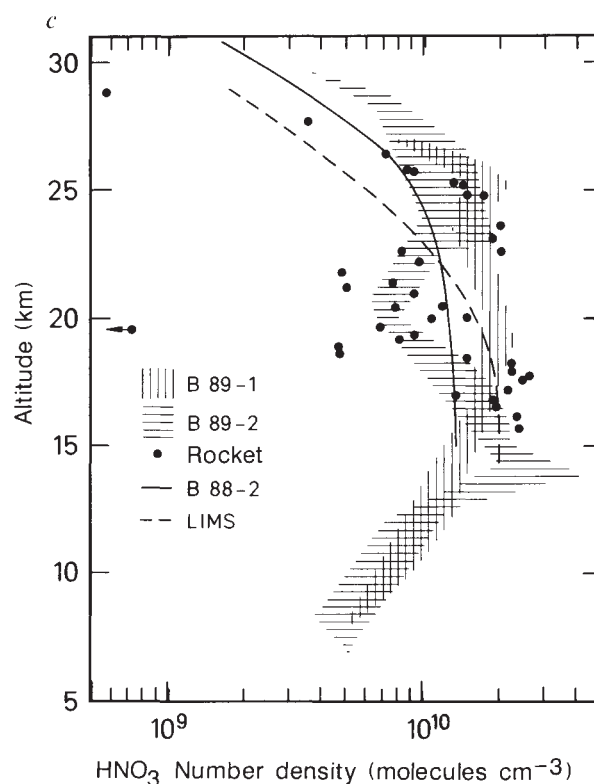
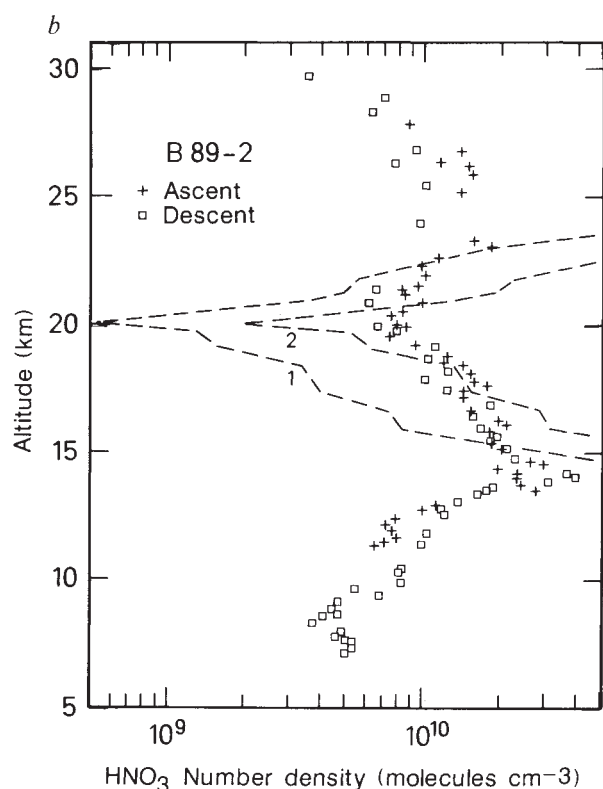


FIG. 2 Number densities of gaseous nitric acid measured by the balloon- and rocket-borne ACIMS instruments. *a*, B89-1 along with a monthly and zonally averaged (January 1979; 70°N) profile measured²⁴ by LIMS (Limb Scanning Infrared Monitor of the Stratosphere). Also shown are two model profiles for the equilibrium saturation nitric acid vapour concentration (HNO_3/NAT over NAT). Model 1 considers the measured temperatures and a water-vapour volume mixing ratio of 5 p.p.m.v. Model 2 considers a temperature being 1° higher and 4 p.p.m.v. For comparison a constant volume mixing-ratio (0.5 p.p.b.v.) profile is also given. *b*, B89-2 data, both measured on 30 January 1989. Also shown are models 1 and 2. *c*, Composite of B89-1, B89-2 and rocket profiles.



water-ice aerosols) followed by sedimentation of HNO_3 -containing aerosols; (3) advection of HNO_3 -poor air. Again, the third possibility is not supported by the ozone measurements made on B89-2. The second possibility is plausible, particularly in view of the HNO_3 excess observed around 14 km. Because of wind shears, however, the column segments could be advected from different regions so that the vertical column encountered by B89-2 might contain excess HNO_3 around 14 km without showing HNO_3 removal around 20 km. On the other hand, it seems that the vertical HNO_3 column segment of the HNO_3 excess (13–16 km) being $\sim 8 \times 10^{15}$ HNO_3 molecules per cm^2 is comparable to the bite-out (18 to 23 km; 9×10^{15} HNO_3 molecules per cm^2), consistent with cause (2) and an unchanged vertical column. The first possibility may also contribute to the bite-out because the layer in which the bite-out occurred was also the coldest layer. Observations of PSCs above northern Scandinavia on 30 January are also evidence for stratospheric NAT aerosols. In the first place the aerosol counter³² flown on B89-2 detected strongly enhanced concentrations of relatively large aerosols with radii in excess of 2–3 μm , particularly in the HNO_3 depletion layer (18–23 km). There were also ground-based visual observations of widespread PSC above Esrange on that day.

Nitric acid condensation

From the measured HNO_3 profile of B89-1, a typical water-vapour profile measured by LIMS^{34,35} (January 1979, 68° N), and the laboratory data¹² we can calculate condensation temperatures for NAT ($T_c(\text{NAT})$) and water ice ($T_c(\text{ice})$) aerosol formation (Fig. 3). The $T_c(\text{NAT})$ values are larger than the measured atmospheric temperature at 18–24 km (B89-1) and 15.5–23 km (B89-2), implying that NAT aerosols may have formed in these layers. By contrast, temperatures did not become lower than $T_c(\text{ice})$ which implies that water ice should not have been present. On 30 January 1989 the temperature was almost equal to $T_c(\text{NAT})$ down to ~ 13 km. On 31 January, one day after B89-2, temperatures became lower than $T_c(\text{NAT})$ at all stratospheric heights below ~ 26 km and lower than $T_c(\text{ice})$ at heights between ~ 14 and 19 km and below 11 km. On that day spectacular PSCs were seen.

We have also calculated equilibrium saturation HNO_3 vapour pressures (converted to HNO_3 concentrations) over NAT using our measured atmospheric temperatures, LIMS- H_2O and the laboratory data¹² (see Fig. 2a, b curves 1). For B89-1 these are much lower than the measured HNO_3 concentrations within the layer (18–24 km). However, within that layer the B89-1 data does not show a substantial decrease of gaseous nitric acid. We note that the calculated equilibrium HNO_3 concentration contains a substantial uncertainty that is introduced mainly by the uncertainties of the water vapour abundance (which was not measured) and the atmospheric temperature (which should be accurate within $\pm 1^\circ$). A temperature change of 1° changes the equilibrium HNO_3 concentration by a factor of about two. The same change can be introduced also by a change of the water vapour abundance from the value taken above (5 p.p.m.v.) to either 6.4 or 3.9 p.p.m.v. Curve 2 in Fig. 2a, b give equilibrium HNO_3 concentrations (lower by a factor of four) considering either an increase of temperature by 2° , a decrease of H_2O to 3.0 p.p.m.v., or a combination of an increase of temperature by 1° and a decrease of H_2O to 3.9 p.p.m.v. For B89-1, curve 2 is hardly lower than the measured HNO_3 profile; for B89-2, curve 2 is still smaller than the measured HNO_3 concentrations. Here it seems that curve 1 reproduces the top of the HNO_3 depletion layer whereas curve 2 reproduces the bottom. Interestingly the layer with excess HNO_3 is located just below the cold layer, or the crossover of curve 1 (Fig. 2b) and the B89-1 profile (Fig. 2a). By contrast, the core of the depletion layer is not reproduced by the models that give lower values. Possible reasons for this discrepancy may be: (1) experimental problems including a memory effect (desorption of HNO_3 from the tube walls, gondola or even the balloon) and partial aerosol evaporation in the flow tube of the ACIMS instrument; (2) an even lower H_2O abundance; (3) non-equilibrium conditions. The second reason is plausible, particularly if the bite-out results from HNO_3 removal through HNO_3 incorporation into water-ice particles in which case also H_2O depletion should have occurred. The third reason is also plausible because the lifetime of a gaseous HNO_3 molecule for sticking to pre-existing $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ aerosols serving as condensation nuclei is of the order of ten hours around 20 km. Hence fluctuations of atmospheric temperatures

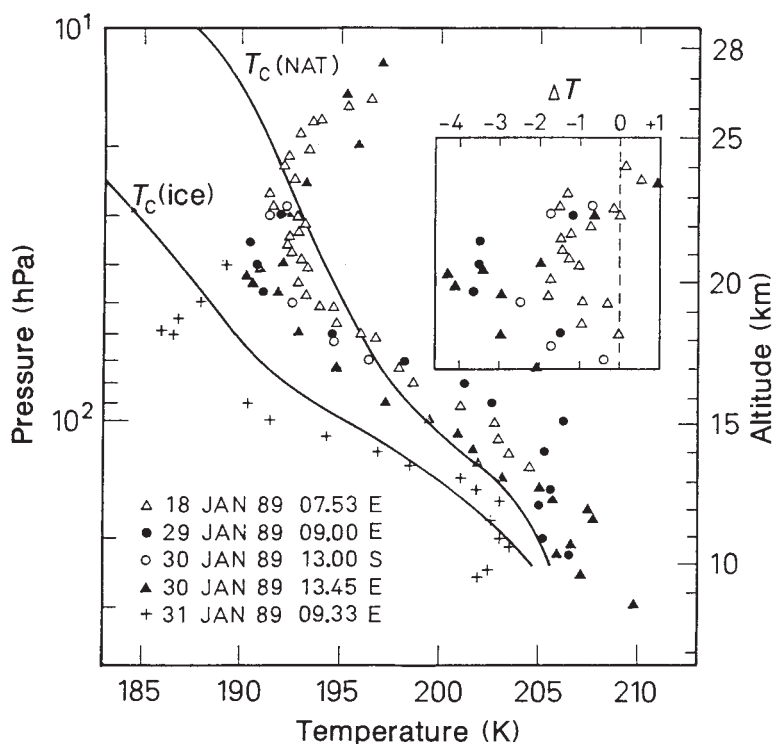


FIG. 3 Stratospheric temperatures measured by balloon-borne sensors launched at Esrange (E) and Sodankila (S) (100 km east of Esrange) on 18, 29 and 30 January 1989. Also given are model profiles for condensation temperatures for NAT ($T_c(\text{NAT})$) and water ice ($T_c(\text{ice})$). The insert gives $\Delta T = T - T_c(\text{NAT})$.

within hours may induce substantial departures from a condensation/evaporation equilibrium. The first possibility cannot be entirely ruled out. It is conceivable that a fraction f of NAT aerosols entering the flow tube of the ACIMS instrument experiences sticking collisions with the tube wall upon which they would quickly evaporate because the tube wall is at a higher ($10\text{--}20^\circ$) temperature than the ambient atmospheric gas temperature. At present we do not know the exact value of f but we expect it to be markedly smaller than one. We note that the length of the flow tube of the rocket instrument was only 50 cm compared to 200 cm for the balloon instrument and that the rocket flow tube was almost linear. We therefore expect f to be smaller for the rocket instrument than for the balloon instrument. In fact, in the HNO_3 depletion layer, the rocket data are systematically lower than the balloon data, consistent with this expectation. This is particularly true for the extremely low upper-limit value at 19.6 km, which was measured by the rocket instrument almost exactly at the same altitude where the model predicts a minimum (20 km). If the bite-out were exclusively caused by HNO_3 removal through sedimentation $T_c(\text{NAT})$ would have decreased by about 2° around 20 km. If H_2O , as well as HNO_3 was being removed a further decrease of $T_c(\text{NAT})$ should have occurred.

Implications

The present measurements of gaseous nitric acid in the Arctic winter polar vortex have interesting implications for the formation and mass of PSCs and their possible effect on ozone concentration. The data indicate that on 30 January 1989, at $\sim 18\text{--}23$ km above northern Sweden, nitric acid vapour underwent condensation with a major fraction ($\approx 80\%$) of the available HNO_3 becoming incorporated into aerosols. This may have led to a total NAT-aerosol surface-area density A of about $2 \times 10^{-8} \text{ cm}^2 \text{ per cm}^3$ which implies a lifetime of a gaseous molecule against a sticking collision with an aerosol surface of ~ 2 h. Considering measured reaction probabilities per collision, γ , ($0.2\text{--}0.02$; ref. 9–11, 14–17) for surface reactions of HCl , ClONO_2 and N_2O_5 , one obtains lifetimes of the above molecules against surface reactions on NAT aerosols of the order of three hours to one day. If the bite-out was mostly due to HNO_3 removal through sedimentation, however, A would be smaller.

Effective condensation temperatures of ~ 193 K around $18.5\text{--}23$ km, are approximately consistent with recent model predictions considering laboratory data¹², the unperturbed (HNO_3) profile (B89-1) and a typical water-vapour volume mixing ratio of 5 p.p.m.v. An inspection of Fig. 1 reveals that, at the centre of the Arctic cold region, temperatures of 30 hPa were lower than $T_c(\text{NAT})$ throughout January. Hence, NAT aerosols should have been present during January. At Esrange, which was located in the vicinity of the usual vortex boundary, temperatures

at the 30-hPa level became lower than $T_c(\text{NAT})$ during four periods (days: 2–4; 17–21; 24–27; 29–33; total: 17 days). For the 50-hPa level the total time span with $T \leq T_c(\text{NAT})$ was only nine days.

By contrast to nitric acid condensation, condensation of pure water vapour was hardly possible. At the 30-hPa level, $T \leq T_c(\text{ice})$ occurred only for two days (1 and 2 February) and only at the cold centre of the vortex. However, small-scale water-ice cloud formation (mother of pearl clouds) induced by orographic waves resulting from strong winds blowing across the Scandinavian mountain ridge were often sighted above Esrange.

It seems that the persistence of NAT aerosols, around 20 km, may have been sufficient to activate chlorine and to passivate reactive nitrogen by surface reactions. If so, the region around 20 km may have been primed for chlorine-catalysed ozone destruction, which starts after the end of the polar night. Even with a full activation of the available chlorine, however, the typical rate of ozone destruction would be only $\sim 0.04\%$ per sunlight hour. Hence, at 20°N , only about 6% of the ozone initially present around 20 km would be destroyed by the middle of February, when the vortex broke down in 1989. In reality a full activation of the available chlorine may not persist sufficiently long because of the re-formation of NO_2 resulting from HNO_3 photolysis. Therefore removal of HNO_3 through sedimentation of HNO_3 -containing aerosols would be very important. If, as suggested by the present data, some HNO_3 removal by sedimentation took place around 20 km the timescale for repassivation of chlorine would have increased. In the extreme case (in which the observed HNO_3 depletion around 20 km was entirely due to sedimentation) the timescale for re-formation of NO_2 and therefore also of repassivation of chlorine would have increased by a factor of about four. During that time substantial ozone destruction may take place if the vortex lives long enough. The latter, however, was probably not the case in 1989.

Natural changes of the meteorology of the winter Arctic stratospheric vortex may induce marked changes of the spatial and temporal extent of NAT aerosols. It is conceivable that long-term changes of NAT aerosols may also be introduced by man's activities. The increase of atmospheric infrared-active gases, which cool the stratosphere by emission of infrared radiation, may lead to a cooling of the stratosphere. The present models³⁶ predict that a cooling of the winter polar vortex by up to about 6° for a doubling of CO_2 may occur in the twenty-first century. Also stratospheric water vapour may increase because of the increase of atmospheric methane the stratospheric photooxidation of which produces at present about half of the stratospheric water vapour. An increase of stratospheric water vapour would increase $T_c(\text{NAT})$ and thereby the spatial and temporal extent of NAT aerosols. □

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Organization of microtubules in dendrites and axons is determined by a short hydrophobic zipper in microtubule-associated proteins MAP2 and tau

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Here we report that the microtubule-associated proteins MAP2 and tau share two separable functional domains. One is the microtubule-binding site which serves to nucleate microtubule assembly; the second is a short C-terminal α -helical sequence which can crosslink microtubules by means of a hydrophobic zipper interaction into dense stable parallel arrays characteristic of axons or dendrites. Thus, interactions between molecules of a single type are capable of drastically reorganizing microtubules and completely suppressing their dynamic properties.

MICROTUBULES are versatile and ubiquitous polymers that play an essential part in a wide range of fundamental cellular events. Both the mitotic spindle and the interphase cytoskeleton are formed from rapidly turning-over microtubule populations with half lives of less than a few minutes, which grow from and shrink towards the microtubule organizing centre(s)¹⁻⁶. In addition to creating such highly dynamic structures, microtubules can also form more stable configurations that are found in many differentiated cell types. Examples include the marginal band, which rings the circumference of platelets, the manchette, which sheathes the nuclei of developing spermatids, ciliar and flagellar axonemes, and the closely packed arrays of microtubules supporting neuronal processes and acting as the tracks for axonal transport. The microtubules that make up all of these widely different static and dynamic organelles are, for the most part, remarkably similar: each is a hollow cylindrical rod consisting of thirteen protofilaments⁷. An important question therefore concerns the means whereby functional and organizational differences among different kinds of microtubule structures are generated and maintained. The study of accessory proteins that bind to various microtubules provides one approach to answering this question.

When microtubules are purified *in vitro* by multiple cycles of assembly and disassembly, the resulting product consists largely of α - and β -tubulin, in addition to several polypeptides that are collectively known as microtubule-associated proteins (MAPs) (for a review, see ref. 8). The number and relative abundance of different MAPs varies according to tissue or cell type. Because the mammalian brain is very rich in microtubules, brain MAPs have been relatively well studied⁹⁻¹², but the function of only one of the six or so well-characterized brain MAPs has been fully established. This MAP is cytoplasmic dynein, which functions as a motor for retrograde axonal transport^{13,14}. MAP2, of relative molecular mass 200,000 (M_r , 200K), and a related family of proteins known as tau (M_r , 36-38 K), are relatively abundant components of brain microtubules that are localized in separate subcellular compartments within neurons:

tau is restricted to axons¹⁵, whereas MAP2 is found largely in dendrites¹⁶. The localization of MAP2 molecules results from the subcellular sorting of their messenger RNA¹⁷. In common with most other MAPs, both MAP2 and tau promote microtubule assembly *in vitro*^{10,12,18}, and tau enhances the stability of microtubules when microinjected into cells¹⁹. Tau is of particular interest because it forms an integral part of the neurofibrillary tangles of Alzheimer's disease (for a review, see ref. 20).

The recent cloning of complementary DNAs encoding MAP2²¹ and tau²² allows a molecular approach to the study of the roles of these proteins in the organization and functioning of the neuronal cytoskeleton. To begin to assess the significance of MAP-tubulin interactions on microtubule behaviour, we performed experiments to study what happens to the microtubules in cultured cells when they interact *in vivo* with MAPs that are normally expressed only in neurons. These experiments show that the expression of MAP2 (and to a lesser extent tau) causes the cells' microtubules to form into bundles that are reminiscent of the long parallel arrays of microtubules found in neuronal dendrites and axons. These bundles are stable against microtubule-depolymerizing drugs and, unlike microtubules in normal cultured cells, are not necessarily nucleated from the centrosome. We show that these bundles result from the crosslinking of microtubules by means of cooperative intermolecular interactions involving a short hydrophobic C-terminal domain present in both MAP2 and tau. This domain has some of the characteristics of a leucine zipper²³⁻²⁵ and can be functionally replaced by the leucine zipper from the yeast DNA-binding protein GCN4. We describe a model, based on our data, for the way in which MAP2 and tau crosslink microtubules. This model accounts for the organization of microtubules in the dendrites and axons of neuronal cells.

MAP2 in tissue-culture cells

To study the consequences of expressing MAP2 in cultured cells, we assembled MAP2-encoding cDNAs²¹ into a single contiguous sequence and inserted this sequence into a mammalian expression vector. The effects of the expression of MAP2 were then monitored in double-label immunofluorescence experiments after transfection into various cell lines. The results of some of these experiments are illustrated in Fig. 1. In most of the transfected cells, the interphase microtubule network was dramatically altered: dense parallel bundles of microtubules appeared that tended to be either relatively straight (for example, Fig. 1a-e) or to form a ring around the circumference of the cell (Fig. 1f). The microtubules in these bundles were not nucleated from the microtubule organizing centre, as are normal interphase microtubules. Bundled microtubules were observed in a wide range of transfected mammalian cells, including HeLa cells, NIH 3T3 cells, L-cells and CHO cells, as well as two neuronally derived lines, R4 and NA.

Cells expressing MAP2 are more intensely labelled with an anti-tubulin antibody, indicating that MAP2 promotes microtubule assembly *in vivo*, as it does *in vitro*. To establish this

experimentally, we made quantitative measurements of the photoemission from 50 individual transfected and nontransfected HeLa cells labelled with an anti- α -tubulin antibody, using a SIT camera and image digitizing equipment. On average, the fluorescent intensity of transfected, detergent-extracted cells was 1.7 ± 0.1 -fold greater than that of nontransfected cells. This is consistent with a shift of the dynamic equilibrium towards microtubule polymerization. As a result of this shift, tubulin synthesis may be up-regulated by means of an autoregulatory mechanism in which the stability of tubulin mRNAs is modulated by the concentration of unpolymerized tubulin²⁷; another possibility is that the tubulin protein itself is stabilized.

As a test of the stability of microtubules bundled by MAP2 *in vivo*, we exposed cells transfected with MAP2 to the microtubule-depolymerizing drugs colchicine and nocodazole. We found that under conditions where the centrosomes are the only surviving microtubules in normal cells, microtubule bundles induced by MAP2 expression remain intact (Fig. 2). This implies that the bundles are stable and do not turn over, because microtubules cannot re-form in the presence of these drugs. To find out whether the microtubule bundles are a result of the increased number and stability of microtubules *per se*, nontransfected cells were treated for 3–36 h with different concentrations of taxol, a drug that promotes microtubule assembly²⁸. Although

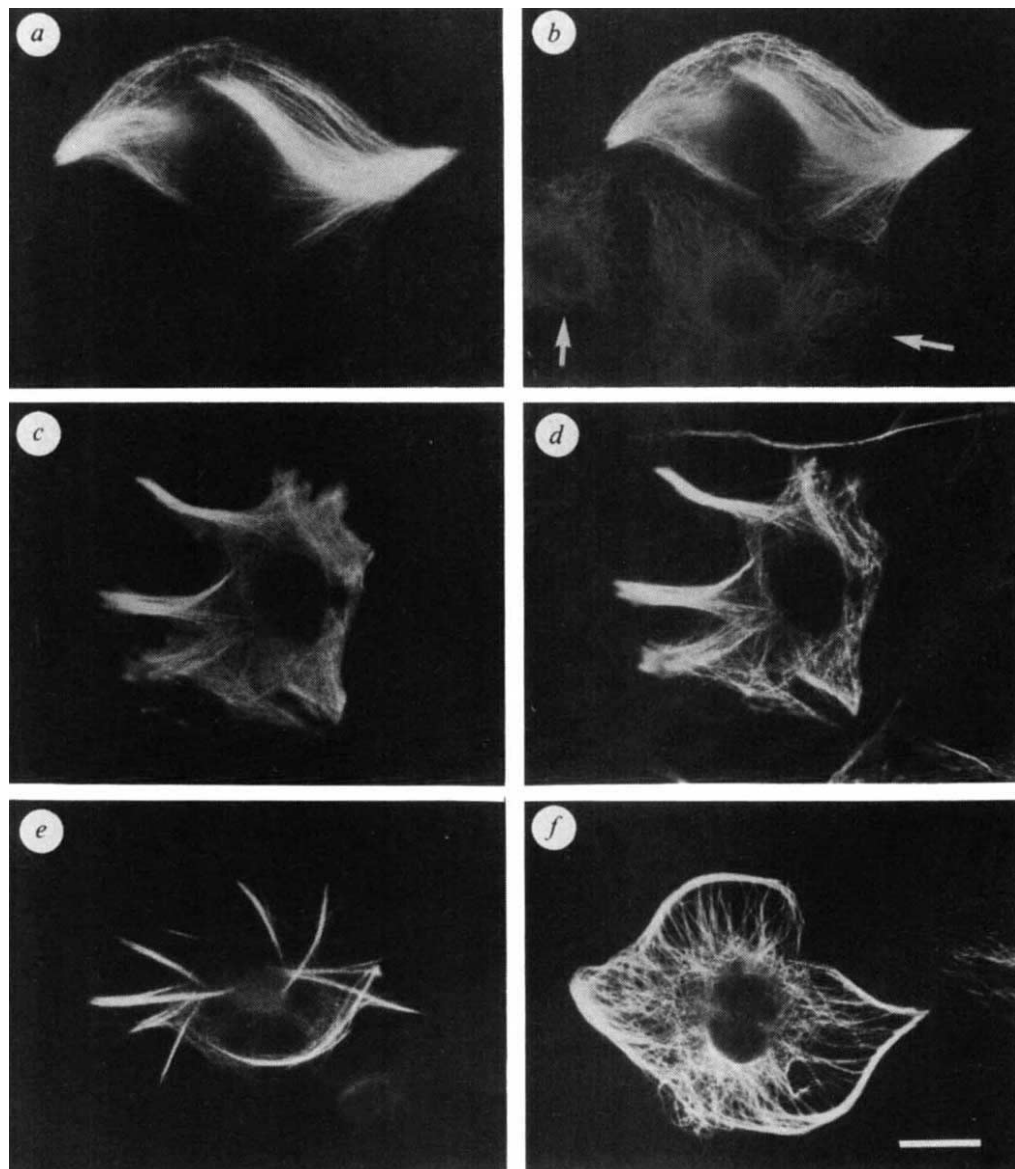
taxol-treated cells contained more microtubules than did non-treated cells, bundles were never observed; rather, exposure to taxol induced a completely different phenotype characterized by fine, dense microtubule arrays (data not shown).

The microtubules in transfected cells expressing MAP2 are not always reorganized into bundles. Indeed, about one-third of MAP2-expressing cells did not contain bundled microtubules detectable by fluorescence microscopy, the presence of conspicuous bundles being qualitatively correlated with the level of MAP2 expression, as judged by fluorescent intensity. Such variations in levels of expressions are to be expected in transient transfection experiments in which different transfected cells take up widely ranging quantities of DNA. Thus, it would seem likely that microtubule bundling by MAP2 is a cooperative phenomenon requiring a threshold level of microtubule-bound MAP2. This threshold level is likely to be exceeded in neurons, because MAP2 is expressed at high levels in whole brain and, moreover, is concentrated in dendrites^{16,17}.

We attempted to generate a MAP2-expressing cell line, using many types of cultured cells and both inducible and non-inducible promoters to drive MAP2 expression. In these experiments, we either obtained clones with no detectable MAP2 expression, or mixed populations containing a proportion of MAP2-positive cells that could not be recovered by recloning to yield a

FIG. 1 Microtubule bundling in tissue culture cells transfected with MAP2. Cells transfected with a construct designed to express MAP2 were analysed by indirect double-label immunofluorescence with an anti-MAP2 antibody and an anti- α -tubulin antibody. *a, b*, HeLa cells; *c, d, f*, NIH 3T3 cells; *e*, L cells. *a, c, e, f*, Anti-MAP2 staining. *b, d*, Same fields as *a* and *c*, respectively, stained with an anti- α -tubulin antibody. Untransfected HeLa cells with a normal microtubule phenotype detected only by the anti- α -tubulin antibody are indicated with arrows in *b*. Scale bar, 10 μ m.

METHODS. A full-length MAP2 cDNA was assembled from overlapping MAP2-encoding cDNA fragments and inserted into the plasmid pSV expression vector⁴⁴. Cells grown on glass cover-slips in DMEM medium containing 5% (HeLa cells) or 10% (NIH 3T3 cells, L cells) FCS were transfected with this construct by the method of Chen and Okayama⁴⁵. Cells were extracted 72 h after transfection with 0.2% Triton X-100 in microtubule stabilizing buffer³³, fixed by immersion in methanol at -20°C for 10 min, rehydrated in phosphate-buffered saline and double labelled with an anti- α -tubulin monoclonal antibody (Amersham) and an anti-MAP2-specific antibody⁴⁶.



homogeneous MAP2-expressing cell line. We conclude that even very low levels of MAP2 are deleterious to cell growth. Because microtubule instability is essential for mitosis, it seems probable that stabilization of spindle microtubules by MAP2 slows or inhibits cell division. This could explain why MAP2 is expressed post-mitotically in the developing brain²⁹.

Microtubule bundling

The encoded amino-acid sequence of MAP2 shares extensive similarity with that of tau protein within the C-terminal 200 amino acids, including a region containing three imperfect 18-amino-acid repeats, each separated by 13 or 14 amino acids²¹. Co-polymerization experiments performed using fragments from within the homologous domain of either MAP2 or tau have shown that this region binds to microtubules *in vitro*^{21,30}. However, these experiments do not define the sequences that are necessary or sufficient for MAP2 binding *in vivo*, nor do they address the issue of microtubule bundling. To delineate the domains within the MAP2 molecule that are responsible for microtubule binding and bundling, we constructed a range of deleted molecules (Fig. 3) and tested their ability to bind to microtubules and/or bundle them in transient transfection assays. Because the MAP2 microtubule-binding domain includes the three imperfect C-terminal repeats, we first

TABLE 1 Quantitative analysis of bundled microtubules in HeLa cells

Construct	% Transfected cells containing bundled microtubules*
MAP2	69 ± 5
FS	75 ± 2
FS + L	32 ± 7
T4	5 ± 1
STS	0

Cells were transfected with MAP2 and tau-encoding constructs (see Fig. 3).

*Standard deviation calculated after examination of four transfection experiments per construct.

examined the consequences of removing this entire domain from the MAP2 molecule (Fig. 3, construct STS). Transfection of the STS construct encoding such a deleted MAP2 protein resulted in the complete abolition of the capacity to bind to microtubules (Fig. 4a, b). This observation is consistent with the C-terminal location of the microtubule-binding domain indicated both by *in vitro* co-polymerization experiments and by the existence of homology in this region between MAP2 and tau molecules.

To determine whether the capacity to bundle microtubules is conferred by sequences 5' to those encoding the microtubule-binding domain, we made several deleted constructs (Fig. 3: KST, SKS, PS, FS). Transfection of each of these four constructs into cultured cells yielded a phenotype indistinguishable from that observed with intact MAP2. Because these deletions collectively encompass virtually the entire N-terminal portion of MAP2 up to amino-acid residue 1,621, we conclude that both the capacity of MAP2 to bind to microtubules and to bundle them resides between amino acids 1,621 and 1,828, that is, in the C-terminal 12% of the molecule.

We next made a set of constructs encoding MAP2 proteins with C-terminal deletions to see whether the binding and bundling properties of this region are located in separable domains. We achieved this by introducing deletions in the FS construct (Fig. 3), because this construct encodes a molecule that (1) yields a phenotype identical to that yielded by intact MAP2, and (2) contains only an N-terminal region (which allows efficient translational initiation and detection with a specific antibody) coupled to the relevant C-terminal region. The smallest deletion removed only 14 C-terminal amino acids (Fig. 3, construct FS-H). Whereas the polypeptide expressed from this construct bound to microtubules (Fig. 4c), no microtubule bundling was observed in any of several experiments using a variety of different cell lines. We conclude that the microtubule binding and bundling properties of MAP2 are conferred by separate domains, and that the ability to form bundles is associated with the extreme C-terminus of the molecule.

The Chou-Fasman and Garnier algorithms^{31,32} both predict that the C-terminal 18 amino acids of MAP2 have a high probability of forming a short α -helix nucleated by a proline residue at position 1,810. In addition, this region is rich in hydrophobic residues that lie on one side of the predicted α -helix, and therefore has the potential to form a coiled-coil interaction analogous to that found in leucine-zipper sequences. Such sequences have been described in several nuclear oncogenes and transcription factors, where they form dimers by means of hydrophobic interactions^{24,25}. Such interactions between C-terminal α -helices of microtubule-bound MAP2 might, therefore, serve to crosslink microtubules into bundles. To test this hypothesis, we assembled a construct (Fig. 3, construct FS + L) in which sequences encoding the authentic 14 C-terminal amino acids of the FS construct were removed and replaced by sequences encoding the 30-amino-acid leucine zipper from the yeast DNA-binding protein GCN4 (ref. 26). When expressed in cultured cells, this chimaeric protein indeed produced microtubule

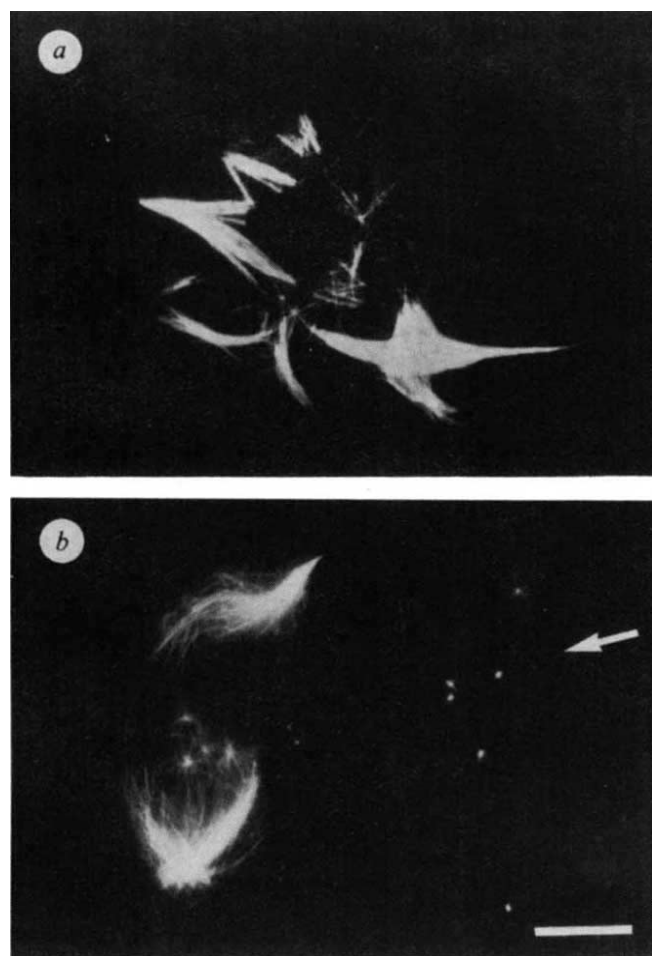


FIG. 2 MAP2-bundled microtubules are stable in the presence of microtubule depolymerizing drugs. Cells transfected with a construct designed to express MAP2 were exposed to colchicine ($2.0 \mu\text{g ml}^{-1}$; a) or nocodazole ($1.0 \mu\text{g ml}^{-1}$; b) for 3 h, and analysed by indirect immunofluorescence with an anti- α -tubulin antibody as described in the legend to Fig. 1. An untransfected cell showing that only the centrosomal cores have survived nocodazole treatment is indicated with an arrow in b. Scale bar, $10 \mu\text{m}$.

bundling (Fig. 4d). The efficiency of bundle formation was somewhat less, however, with the FS+L construct than it was with intact MAP2 or the FS construct. This is probably because the strength of the GCN4 leucine zipper is greater than the strength of the short α -helical C-terminus of MAP2: molecules containing the GCN4 sequence are more likely to form stable dimers before microtubule binding, and hence will have a greater tendency to bind to the same microtubule than to form cross-bridges between different microtubules. We also examined the behaviour of a synthetic polypeptide corresponding to the 22 C-terminal residues of MAP2. Under native gel filtration conditions, this polypeptide migrated with an M_r of approximately twice that of the same material run under fully denaturing conditions (S.A.L. and N.J.C., manuscript in preparation). In summary, the C-terminal 18 amino acids of MAP2 (1) are required for microtubule bundle formation, (2) are predicted to form a hydrophobic α -helix, (3) can be functionally replaced by a domain that is known to dimerize, and (4) behave as a dimer *in vitro*. We conclude that the dimerization of this α -helical domain is alone responsible for the cooperative crosslinking of microtubules into bundles.

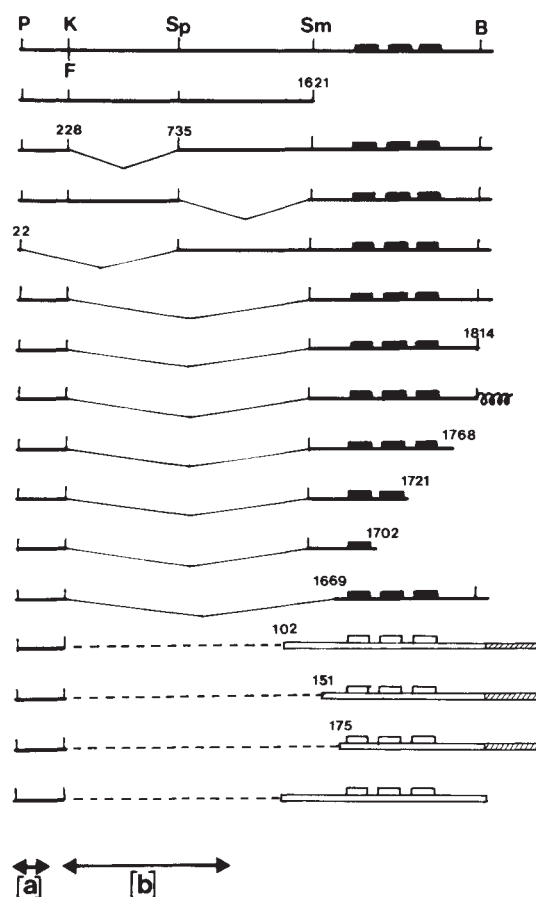
Microtubule binding

The first five deletion constructs shown in Fig. 3 serve to localize the microtubule-binding domain of MAP2 to the C-terminal 207 amino acids. To see if all of this region is required for micro-

tubule binding, we made several additional constructs with deletions either 5' or 3' to sequences encoding the three imperfect repeats. We assayed the binding of the resulting deleted MAP2 proteins to microtubules by immunofluorescence of transfected cells fixed in two different ways: (1) after extraction of the cells with detergent in a microtubule stabilizing buffer³³, and (2) directly with cold methanol, in which case proteins are extracted and fixed concurrently. Of the constructs with deletions 3' to sequences encoding the first 1,621 amino acids, only the protein encoded by the construct FS-H (Fig. 3), which lacks 14 C-terminal amino acids, bound to microtubules in qualitatively the same way as did intact MAP2 (Fig. 4c). Thus, the α -helical C-terminal domain is not part of the microtubule-binding site. Construct FS-C, which lacks sequences encoding the last 59 amino acids of MAP2, expresses a protein which bound to microtubules in a way that was qualitatively different from that of MAP2: in cells extracted with detergent before fixation, protein was found only in regions of high microtubule density (that is, around the microtubule organizing centre (Fig. 4e)), implying a shift in the binding equilibrium away from the bound state. By contrast, MAP2 remained bound to microtubules even after detergent extraction which removes unbound MAP2, implying an equilibrium that strongly favours microtubule binding. Larger deletions from the 3' end (Fig. 3, constructs FS2, FS1) resulted in proteins which did not remain bound to microtubules in detergent-extracted cells; but that proportion of the protein

FIG. 3 Ability of MAP2 and tau-encoding constructs to bind and bundle microtubules. Constructs (not drawn to scale) used in transfection experiments are shown as thick lines (encoding MAP2) or open lines (encoding tau). Raised solid boxes and raised open boxes represent the three imperfect repeats that form part of the microtubule binding site within the homologous region of MAP2 and tau, respectively. A helix attached to the protein encoded by the FS+L construct denotes the leucine-zipper domain derived from the yeast GCN4 DNA-binding protein²⁶. The amino acid residue numbers at the deletion end-points of each construct are shown. Hatched areas represent the extended C-terminus of the long isoform of tau protein²². Horizontal arrows show regions of MAP2 contained in bacterial fusion proteins; one fusion protein was used as an immunogen for the generation of an antiserum specific for the N-terminal 161 amino acids of the MAP2 molecule; the second (clone 59A, encoding amino acids 375–1202; ref. 46) was used as a substrate for the affinity purification⁴⁷ of an anti-MAP2 antiserum⁴⁶. The corresponding position of some salient restriction sites used in making deleted MAP2 constructs are shown (B, *Bgl*I; P, *Pvu*II; K, *Kpn*I; F, *Fsp*I; Sp, *Spe*I; Sm, *Sma*I). The ability of the protein expressed from each construct to bind to microtubules or to bundle them in transfected cells is indicated. Transfected cells were examined both with and without detergent extraction before fixation; this allowed a qualitative assessment of the microtubule-binding properties of the various expressed proteins (see text). For microtubule binding: ++, Protein remains bound to detergent-extracted cytoskeletons; +, Some protein remains bound to detergent-extracted cytoskeletons, but only in regions of high microtubule density; $\pm$, Protein does not remain bound after detergent extraction, but is detectably bound in cells fixed directly; –, No binding. For microtubule bundling: ++, about 70% of transfected cells contain obvious bundles; +, about 30% of transfected cells contain bundles; $\pm$, <10% of transfected cells contain bundles; –, no bundles (see Table 1). Examples of several of these phenotypes are shown in Fig. 4.

METHODS. Deleted constructs were inserted into the plasmid pSV



Construct Binding Bundling

MAP2	++	++
STS	–	–
KST	++	++
SKS	++	++
PS	++	++
FS	++	++
FS-H	++	–
FS+L	++	+
FS-C	+	–
FS2	$\pm$	–
FS1	$\pm$	–
FS3	$\pm$	–
T1	+	–
T2	$\pm$	–
T3	$\pm$	–
T4	$\pm$	$\pm$

expression vector⁴⁴ and introduced into HeLa, NIH 3T3, and various other cell lines by transfection as previously described⁴⁵. Cells were fixed 72 h after transfection, by immersion in methanol at -20°C for 10 min either directly or after extraction with 0.2% Triton X-100 in microtubule-stabilizing buffer³³. Fixed cells were double labelled with an anti- α -tubulin antibody in addition to one of the two anti-MAP2 antisera ([a] or [b]).

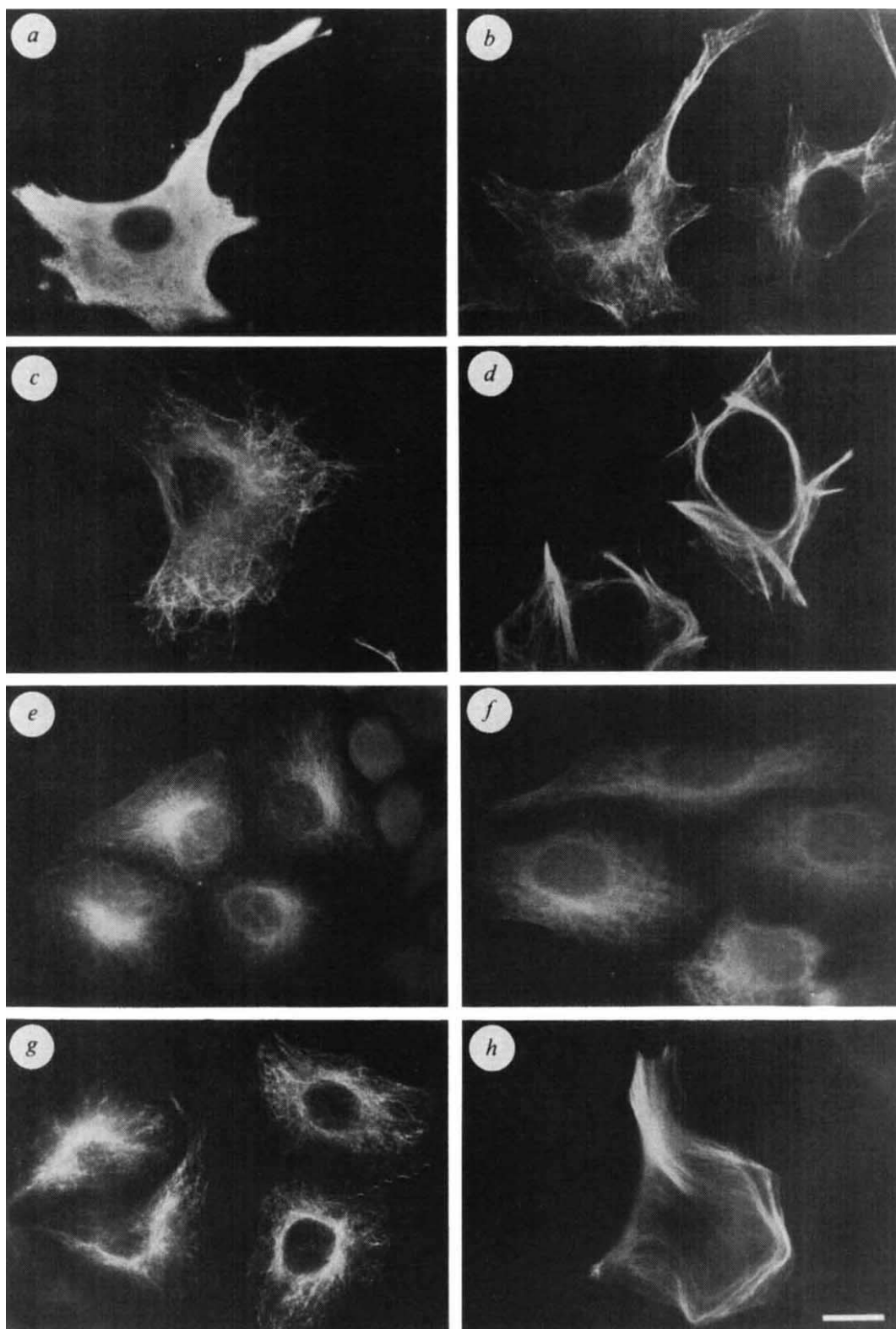
which did bind to microtubules could be detected in cells fixed with methanol (Fig. 4f). Thus, removal of these sequences considerably diminishes microtubule binding. Finally, the construct FS3, in which sequences 5' to the three repeats are deleted, produced a protein with binding properties similar to those of the proteins encoded by constructs FS2 and FS1. We conclude from these data that the MAP2 domain responsible for microtubule binding *in vivo* encompasses more than the three repeats; sequences on either side of these repeats seem to significantly

contribute to the interaction. It is of interest that the protein encoded by construct FS3, which contains the C-terminal hydrophobic domain but is extractable with detergent, failed to bundle microtubules; this is further evidence for the cooperative nature of the bundling reaction.

To compare the microtubule binding and bundling properties of tau with those of MAP2, sequences encoding the homologous domain from tau were joined in-frame in three places (Fig. 3, constructs T1, T2, T3) to the 5' end of the sequence encoding

FIG. 4 Expression of deleted MAP2 and tau proteins in cultured cells. The deleted constructs shown in Fig. 3 were expressed after transient transfection into HeLa or NIH 3T3 cells. *a*, *b*, Immunofluorescence of cells expressing construct STS examined by indirect double labelling with an anti-MAP2 antibody ([*a*] in Fig. 3) (*a*) and an anti- α -tubulin antibody (*b*). *c*–*h*, Immunofluorescence of cells expressing construct FS-H, FS+L, FS-C, FS2, T1 or T4, respectively, examined by indirect immunofluorescence with the same anti-MAP-2 antibody used to detect the expression of construct STS. Scale bar, 10 μ m.

METHODS. Constructs were introduced into cells by transfection and the cells fixed and labelled as described in the legend to Fig. 3. Cells transfected with construct STS, FS2, T1 or T4 were fixed directly in methanol; cells transfected with construct FS-H, FS+L or FS-C were fixed in methanol after detergent extraction.



MAP2 (which provides for correct translational initiation and recognition of the resulting protein with the anti-MAP2 antibody that we used, see Fig. 3, [a]). Only protein expressed from construct T1 remained bound to microtubules in detergent-extracted cells, indicating that the 50 amino acids preceding the three repeats contribute to microtubule binding. None of these three proteins forms microtubule bundles (Fig. 3). The proteins encoded by constructs T1, T2 and T3, however, derive from a form of tau that contains a C-terminal extension of 25 amino acids, derived from a differentially spliced tau mRNA²². These amino acids are not predicted to extend the hydrophobic α -helix of tau that is 83% homologous to the one in MAP2, but rather form a β -sheet structure. To see whether the form of tau which is coterminal with MAP2 has different bundling properties from the extended form of tau, we made and tested the construct

T4 (Fig. 3). In contrast to construct T1, the expression of construct T4 resulted in some microtubule bundling, even though the binding properties of T4 differ from those of T1 (Fig. 3). These experiments indicate that the C-terminal extension present in some forms of tau contribute to microtubule binding, while at the same time reducing or eliminating bundling, and indicate that effective bundling depends on the C-terminal location of the hydrophobic α -helix. Several other isoforms of tau, which also arise by differential splicing³⁴, differ in their microtubule-binding domains: they contain four as opposed to three imperfect repeats, and thus would be expected to bind to microtubules more strongly than do the proteins encoded by constructs T1 and T4 described here. Because for MAP2 we have shown that bundling is dependent on unimpaired binding, we predict that these isoforms can bundle microtubules more efficiently than can the T4-encoded protein. Thus, differential splicing of the tau gene is likely to lead to microtubules that are bundled and stabilized to different extents. These differences between tau and MAP2 and between different forms of tau probably reflect different requirements for the cytoskeletons of axons and dendrites during development.

Bridging to neurofilaments

MAP2 has been reported to form cross-bridges between microtubules and neurofilaments^{35,36}, the two cytoskeletal polymers that run parallel in neuronal processes, and there is evidence for the binding of MAP2 to neurofilaments *in vitro*³⁷. To study this phenomenon, we cotransfected neuronally-derived cell lines (NA, R4) with the construct encoding MAP2 and either of the gene encoding neurofilament proteins NF-L or NF-M^{38,39}. In doubly transfected cells, neurofilament networks and the MAP2-decorated microtubules occupied entirely different regions of the cytoplasm (Fig. 5). Thus MAP2 cannot directly link microtubules to neurofilaments, although this experiment does not rule out the possibility that both MAP2 and neurofilaments bind to another crosslinking molecule that is not expressed in cultured cells.

Microtubules in axons and dendrites

We present a model for the interaction of MAP2 with microtubules (Fig. 6a). MAP2 binds to microtubules by means of a domain that includes, but is not limited to, the three imperfect 18-amino-acid repeats. Because one or two repeats alone bind weakly to microtubules (Fig. 3), and because the spacing of the repeats is consistent with the spacing of subunits within the polymer, each repeat probably interacts with adjacent tubulin molecules. Each MAP2 molecule is depicted as binding along a single protofilament. The long N-terminal arm of MAP2 extends from the microtubule surface, where it is available for interaction with other cellular components, including (among others) associated protein kinases⁴⁰. Each α -helix at the extreme end of the shorter C-terminal arm is shown forming a cross-bridge with another MAP2 molecule. As the experiments described here demonstrate, it is through cooperative interactions of these hydrophobic zipper-like α -helices that bundles of microtubules are formed. The spacing of the microtubules in the bundles is depicted as 40 nm in Fig. 6a, in part for clarity, and in part because this is an upper limit for the length of the cross-bridges assuming a fully extended C-terminus of MAP2. Our model applies equally well to tau protein, which has closely similar microtubule binding and bundling domains. The main difference between tau- and MAP2-induced microtubule bundles is in the length and functional properties of the N-terminal arms of the respective proteins. In addition, we have shown that the manner in which two isoforms of tau protein bind to and/or bundle microtubules is qualitatively different from that of MAP2; this is reflected in the relative size and stability of the bundles.

Our model explains the configuration of microtubules in the axons and dendrites of neuronal cells which, like the micro-

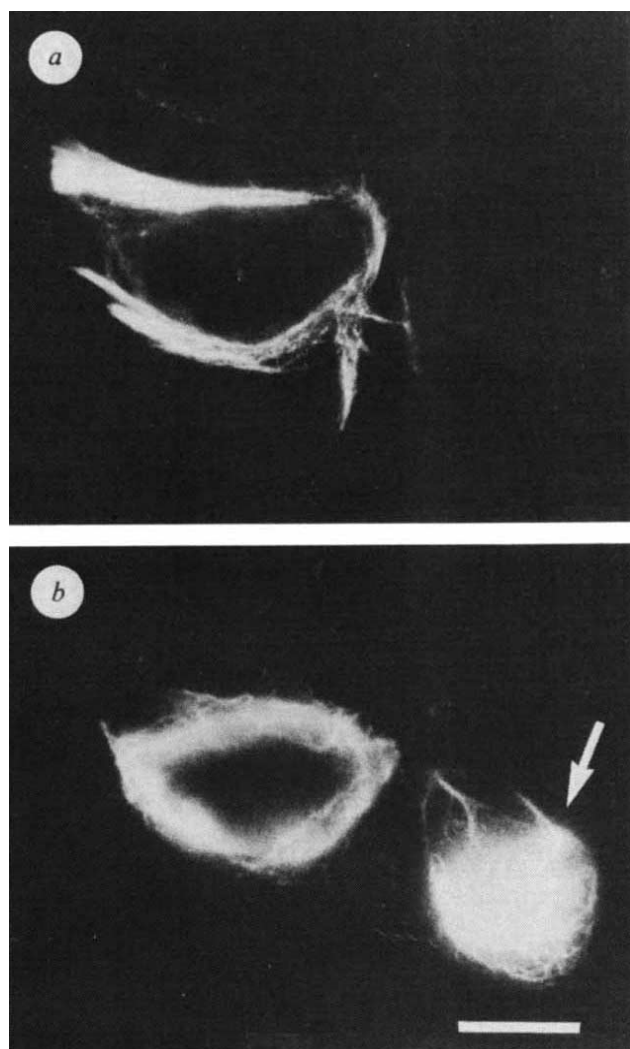
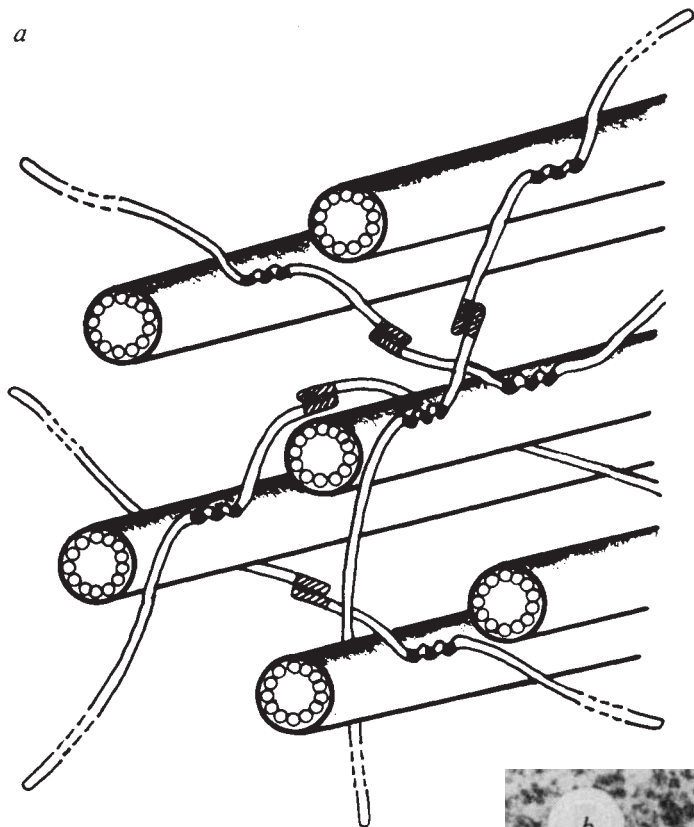


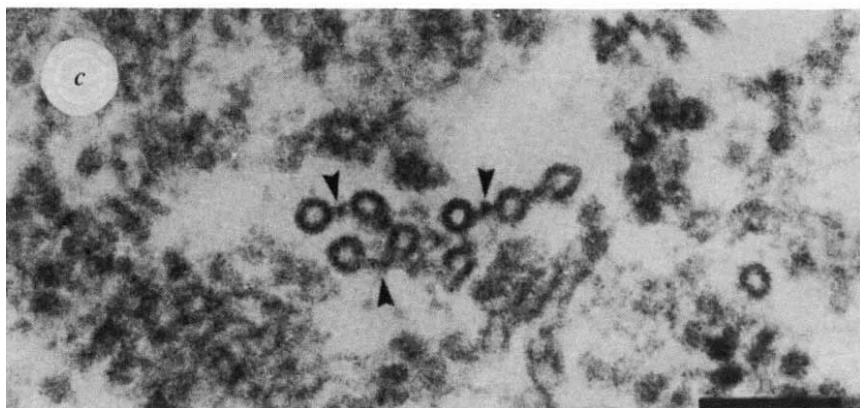
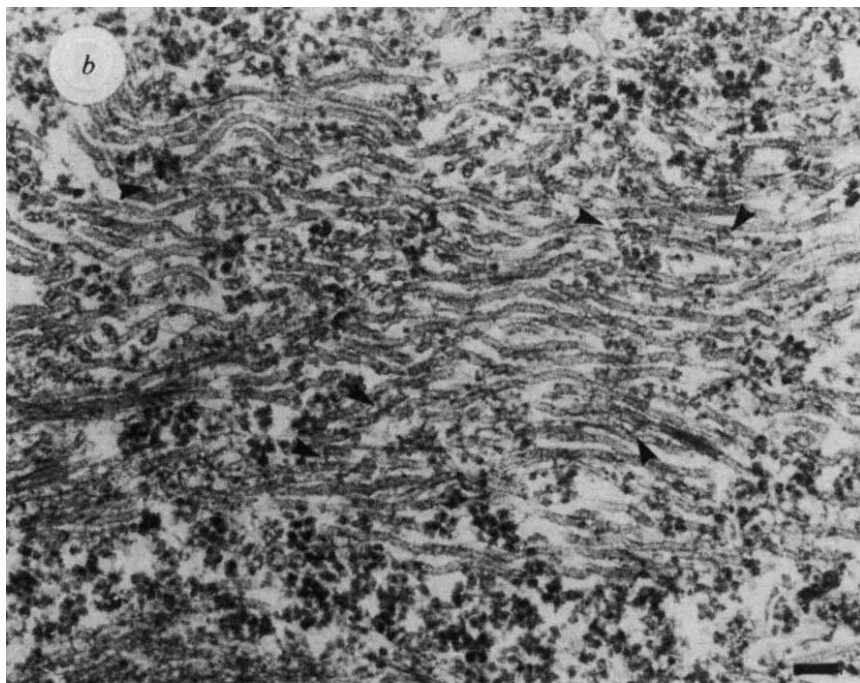
FIG. 5 Co-expression of MAP2 and neurofilament proteins. A neuronally derived cell line (R4) was cotransfected with sequences encoding MAP2 and the low M_r neurofilament protein, NF-L³⁸. Transfected cells were examined by indirect double-label immunofluorescence using a MAP2-specific antibody and a mouse monoclonal antibody specific for NF-L. *a*, Anti-MAP2 antibody; *b*, same field as *a* detected with the anti-NF-L antibody. A cell expressing only NF-L is indicated by an arrow in *b*. Scale bar, 10 μ m. Similar results were obtained in cotransfection experiments with MAP2 and NF-M³⁹ (data not shown).

METHODS. The NF-L gene and the construct expressing MAP2 were cotransfected⁴⁵ into R4 cells. Cells were extracted 72 h after transfection with Triton X-100, fixed in cold methanol, rehydrated and reacted with anti-sera as described in the legend to Fig. 1.

a

tubules in MAP2 or tau transfected cells, are stabilized and bundled into long parallel arrays. Cross-bridges of ~ 20 nm which seem to link microtubules have been observed by freeze-etch electron microscopy in both axons and dendrites and in microtubule pellets⁴¹⁻⁴³; the average distance between microtubules measured in thin sections of dendrites is ~ 100 nm (ref. 48). As a direct test of our model and of the relevance of our transfection experiments to the arrangement of microtubules in axons and dendrites, we examined cells transfected with the FS construct in the electron microscope (Fig. 6*b, c*). This construct was selected for these experiments for ease of interpretation: the protein that it encodes binds to and bundles microtubules in a manner indistinguishable from intact MAP2 (Fig. 3), but lacks all but a very small segment of the long N-terminal arm. Different sized bundles of microtubules were present in the cytoplasm of transfected cells (Fig. 6*b*). The appearance of these bundles was not affected by treatment with colchicine or nocodazole. Numerous cross-bridges with a maximum length of ~ 25 nm were observed between neighbouring microtubules in the bundles. These cross-bridges correspond in size with those observed in dendrites and axons; indeed, in some cases, the bridges had a central region of thickening (Fig. 6*c*), which probably reflects the overlap of interacting C-terminal hydrophobic zippers of MAP2 proteins. Although the average spacing between bundled microtubules is less in transfected cells than

FIG. 6 *a*, Model of the organization of microtubules into parallel arrays by self-interaction between the C-terminal α -helices of MAP2 molecules. Microtubules are shown as closely spaced parallel rods made up of 13 protofilaments. MAP2 molecules are shown bound to microtubules by means of the domain including the three imperfect repeats (solid areas); the long N-terminal arms extend among and away from the bundled microtubules. Self-interacting C-terminal α -helical regions of MAP2 molecules are shown (hatched areas) that serve to organize the microtubule bundle. *b, c*, Electron microscopy of bundled microtubules in a transfected HeLa cell. HeLa cells transfected with the FS construct (see text and Fig. 3) were fixed and processed for transmission electron microscopy as described previously⁴⁸. Prominent bundles of interweaving microtubules (*b*) cross-bridged by thin filaments (arrowheads) were evident in transfected cells. In some cases (*c*), more dense segments were present in the middle of these cross-bridges (arrowheads). Scale bar, $0.1 \mu\text{m}$.



in dendrites, this could be a consequence of the relatively high level of expression, with a consequent tightening of microtubules within the bundle. In conclusion, the experiments described here demonstrate that the expression of a single type of molecule

can drastically alter the arrangement and functioning of microtubules, changing a highly dynamic network that grows out from and shrinks towards the centrosome into static stable bundles. □

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Specific proteolysis of the c-mos proto-oncogene product by calpain on fertilization of *Xenopus* eggs

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The *Xenopus c-mos* proto-oncogene product, pp39^{mos}, accumulates in the unfertilized egg during maturation, is hyperphosphorylated and exhibits protein kinase activity. On fertilization, or soon after the completion of meiosis, the accumulated pp39^{mos} undergoes selective proteolysis. Using an *in vitro* protease assay system, we show here that this specific proteolysis is caused by the calcium-dependent cysteine protease, calpain.

IN most species, oocyte maturation is triggered by hormonal stimuli and is followed by the activation of maturation (or M-phase) promoting factor (MPF) which causes germinal vesicle breakdown (GVBD), chromosome condensation and spindle formation in maturing oocytes¹. The MPF activity rises before GVBD and falls after the first meiotic metaphase (metaphase I)². The activity rises again during meiosis II and is stabilized at high levels at metaphase II in fully matured oocytes (or unfertilized eggs) by a calcium-sensitive factor known as cytosolic factor (CSF)^{2,3}. MPF, originally found in the unfertilized amphibian egg⁴, is now known to be ubiquitous in eukaryotes and promotes G2-M transitions in both meiosis and mitosis^{1,5,6}. This factor has recently been shown to consist of at least two components, p34^{cdc2} kinase^{7–9} and cyclin^{10–12}. During both meiosis and mitosis, the inactivation of MPF at the metaphase-anaphase transition has a strong correlation with the phosphorylation of p34^{cdc2} kinase^{13,14} and the degradation of associated cyclins^{10,11,15}.

The *c-mos* proto-oncogene, the cellular homologue of the viral oncogene (*v-mos*) of Moloney murine sarcoma virus¹⁶, is expressed at high levels in germ cells of various vertebrates^{17–21}. *Xenopus c-mos* RNA is expressed as a maternal mRNA in oocytes and persists in early embryos²². The synthesis of *c-mos* product pp39^{mos} is, however, induced only during progesterone activated oocyte maturation²² and this induction precedes both activation of MPF and GVBD²³. When translation of the *c-mos* RNA is specifically blocked with *mos*-specific antisense oligonucleotides, both activation of MPF and oocyte maturation are inhibited^{22,23}. By contrast, when immature oocytes are injected with synthetic *mos* RNA, MPF is activated and oocytes undergo maturation (GVBD) in the absence of progesterone²³. From these results, we have proposed that pp39^{mos} may qualify as a candidate 'initiator'²⁴ of MPF²³. Its activity during maturation is conserved in vertebrates as expression of the mouse *c-mos* product is also required for normal oocyte maturation²⁵.

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In the present study, we show that pp39^{mos} is continuously synthesized during maturation and accumulates in the unfertilized egg as a hyperphosphorylated molecule that has an intrinsic protein kinase activity. We also show that pp39^{mos} stored in unfertilized eggs is selectively degraded at fertilization or at the completion of meiosis by calpain, the endogenous calcium-dependent cysteine protease. Together with results from the accompanying study²⁶, the present results strongly indicate that in early development Mos protein functions as a kinase in a meiosis-specific manner, presumably for stabilization of MPF activity.

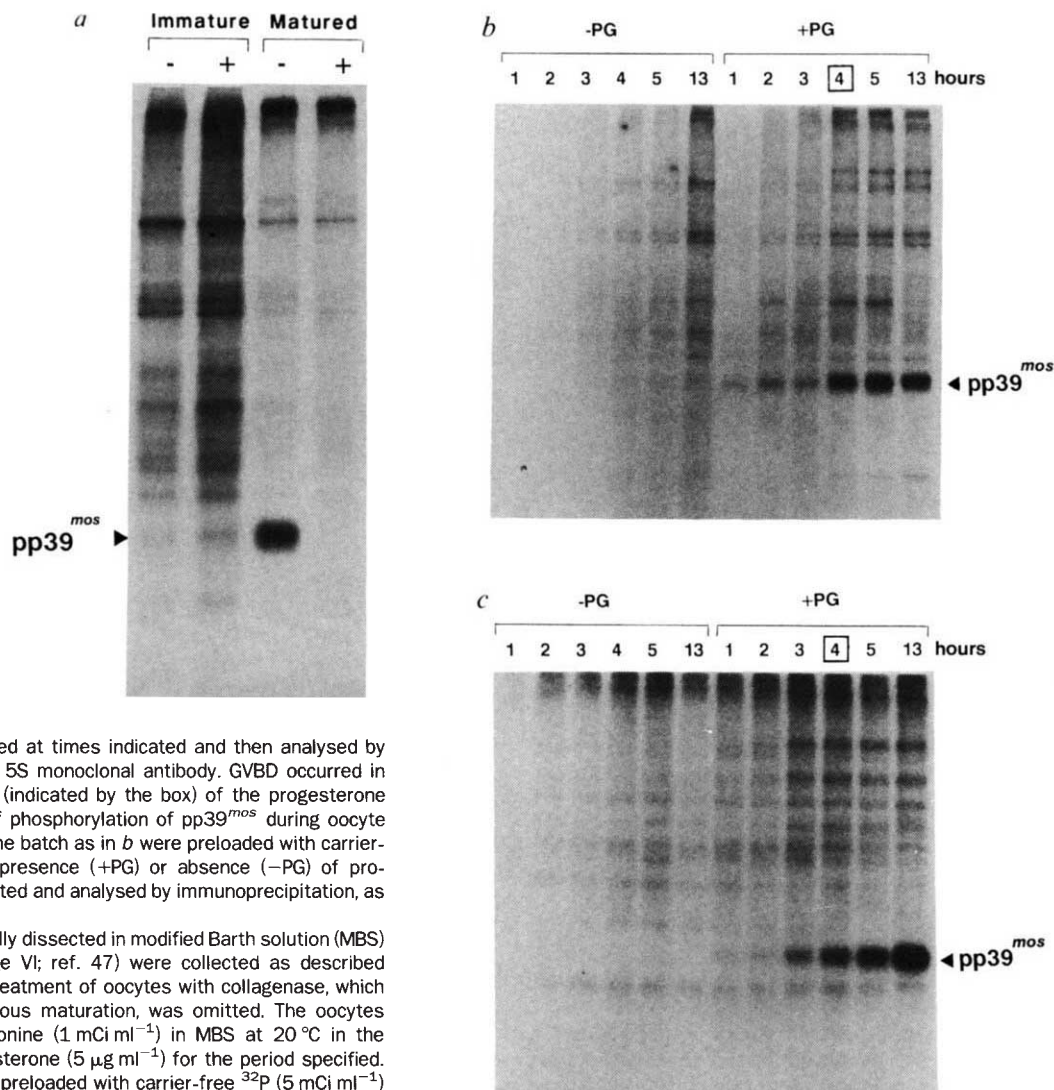
Synthesis and modification of Mos

We used a monoclonal antibody (termed 5S; ref. 23) prepared against bacterially expressed *Xenopus* Mos²². Consistent with previous results using Mos-specific polyclonal antibodies²², Mos protein (pp39^{mos}) was detected only in the progesterone-treated, fully matured oocytes continuously labelled with [³⁵S]methionine for 13 h (Fig. 1a). We discovered that the 5S monoclonal antibody was directed against an epitope in the C-terminal

22-amino-acid sequence of *Xenopus* pp39^{mos} (Fig. 1a) and this peptide was used in subsequent experiments as a competing antigen.

Although pp39^{mos} is required during *Xenopus* oocyte maturation²², its synthesis throughout this period has not been characterized. For this purpose oocytes were continuously labelled with [³⁵S]methionine in the presence or absence of progesterone, collected at appropriate time intervals and subjected to immunoprecipitation analysis with the 5S monoclonal antibody. Without progesterone treatment, no detectable synthesis of pp39^{mos} was observed throughout the cultivation period (Fig. 1b). As demonstrated previously in progesterone-treated oocytes²³, pp39^{mos} is readily detected 1 h after the hormone treatment (Fig. 1b). The level of incorporation continues to increase for 4 h and through GVBD after which it remains high until the completion of maturation (13 h). These analyses also reveal a slight decrease in the apparent mobility of pp39^{mos} with time (Fig. 1b), presumably due to post-translational modification. As pp39^{mos} is phosphorylated in fully matured oocytes²², we suspected that this modification was responsible

FIG. 1 Synthesis and modification of Mos protein (pp39^{mos}) during oocyte maturation. a, Immunoprecipitation analysis of pp39^{mos} in immature and *in vitro*-matured oocytes. Oocytes were metabolically labelled with [³⁵S]methionine for 13 h in the presence (Matured) or absence (Immature) of progesterone and subjected to immunoprecipitation analysis using the 5S monoclonal antibody in the presence (+) or absence (−) of a competing synthetic peptide. The polypeptide(s) in immature oocytes that apparently comigrates with pp39^{mos} is not Mos because it is not competed by the synthetic peptide. b, The time course of synthesis and accumulation of pp39^{mos} during oocyte maturation. Oocytes were labelled continuously with [³⁵S]methionine in the presence (+PG) or absence (−PG) of progesterone, collected at times indicated and then analysed by immunoprecipitation using the 5S monoclonal antibody. GVBD occurred in 50% of the oocytes after 4 h (indicated by the box) of the progesterone addition. c, The time course of phosphorylation of pp39^{mos} during oocyte maturation. Oocytes of the same batch as in b were preloaded with carrier-free ³²P and cultured in the presence (+PG) or absence (−PG) of progesterone. Oocytes were collected and analysed by immunoprecipitation, as in b.



analysed by SDS-PAGE as described²². In some experiments, a synthetic peptide that corresponds to the C-terminal 22-amino-acid sequence (CTGGPPSCSPESNAPPPLGTGL; ref. 22) of *Xenopus* pp39^{mos} was included (10 µg ml^{−1}) in the immunoprecipitation reaction for competition. This synthetic peptide completely blocked the immunoprecipitation of *E. coli*-produced Mos protein by the 5S monoclonal antibody (data not shown).

for the mobility shift. To test this, oocytes were preloaded with carrier-free ^{32}P before treatment with progesterone. Figure 1c shows that incorporation of ^{32}P into pp39^{mos} progressively increased during the course of maturation and although the incorporation was very similar to the incorporation of [^{35}S]methionine, the product in fully matured oocytes (13 h incubation) was more extensively phosphorylated or hyperphosphorylated.

Properties of pp39^{mos} in fully matured oocytes

The existence of hyperphosphorylated pp39^{mos} in fully matured oocytes (or unfertilized eggs) prompted us to examine its biochemical properties. First, we studied the stability of pp39^{mos} in *in vitro*-matured, as well as maturing, oocytes that had previously been labelled with [^{35}S]methionine. In maturing oocytes treated with cycloheximide 30 min before GVBD and incubated

for an additional 2 h, pp39^{mos} completely disappeared (Fig. 2a). By contrast, in fully matured oocytes no detectable degradation of pp39^{mos} occurred during the 2-h incubation in the presence of cycloheximide. These results show that pp39^{mos} is much more stable in matured oocytes than it is in maturing oocytes.

A minute amount of viral Mos protein (0.0005% of total protein²⁷) is present in transformed mouse cells. To determine the amount of the hyperphosphorylated and stable pp39^{mos} in fully matured oocytes, as many as several hundred oocytes were directly homogenized in a standard immunoprecipitation buffer and subjected to immunoprecipitation followed by western blot analysis. In many early attempts, no pp39^{mos} was detected. We found that this was due to its degradation during the mass preparation and that this could be prevented when performed in the presence of the calcium-specific chelator EGTA (data not shown). We therefore employed a special protocol developed

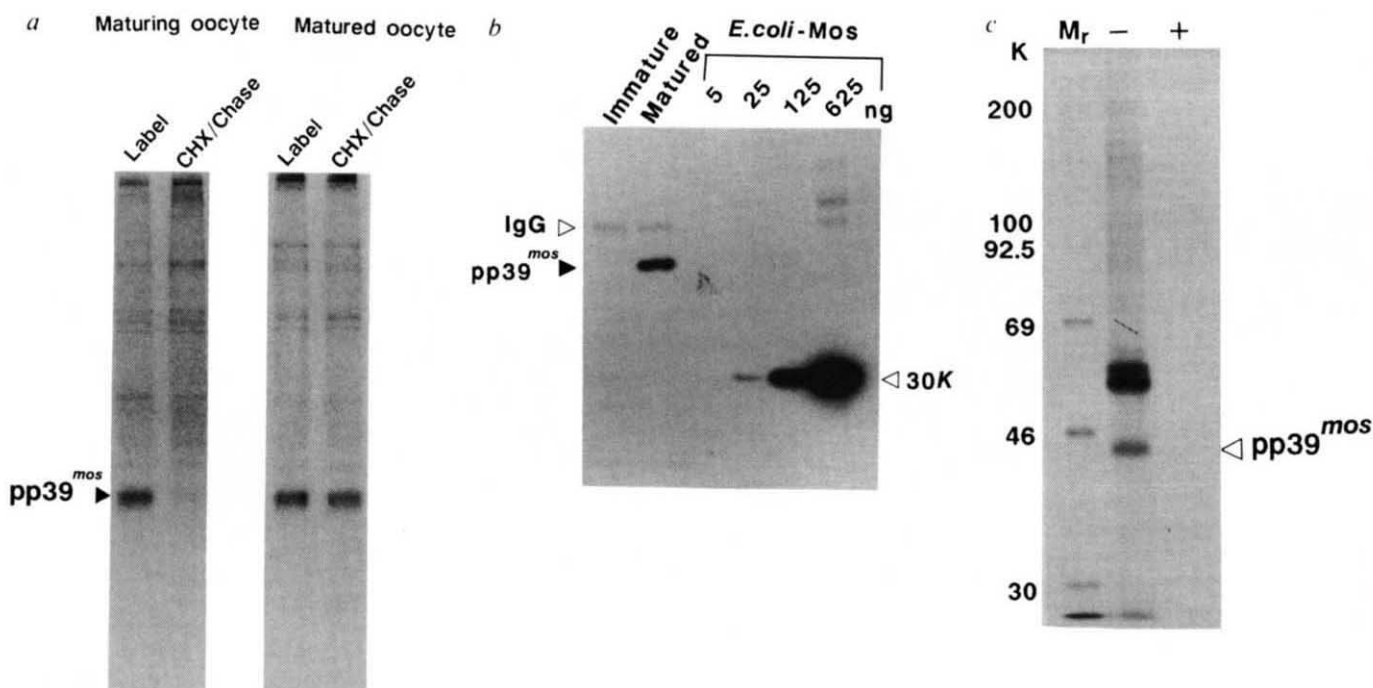


FIG. 2 Stability, amount and kinase activity of pp39^{mos} in fully matured oocytes. *a*, Stability of pp39^{mos} in *in vitro* maturing and matured oocytes. Fully grown oocytes obtained as described in Fig. 1 were labelled continuously with [^{35}S]methionine (1.25 mCi ml⁻¹) in the presence of progesterone (5 $\mu\text{g ml}^{-1}$). Approximately 30 min before GVBD (or 2.5 h after progesterone addition), an aliquot was taken (Label, Maturing oocyte). Another aliquot was washed and chased for 2 h in the presence of cycloheximide (100 $\mu\text{g ml}^{-1}$) (CHX/Chase, Maturing oocyte); oocytes of this group all underwent GVBD about 30 min after the cycloheximide addition. For fully matured oocytes, an aliquot was taken 13 h after the progesterone treatment (Label, Matured oocyte). Another aliquot was washed and chased for 2 h in the presence of cycloheximide (300 $\mu\text{g ml}^{-1}$) (CHX/Chase, Matured oocyte). All the aliquots were then subjected to immunoprecipitation using the 5S antibody as described in Fig. 1. In both maturing and matured oocytes treated with cycloheximide, protein synthesis was inhibited by more than 95% within 30 min after the treatment. *b*, Quantitation of pp39^{mos} in fully matured oocytes. Cytosol from 600 *in vitro* matured as well as immature oocytes was immunoprecipitated with the 5S monoclonal antibody and subjected to western blot analysis using the same antibody. Serial dilutions of truncated *Xenopus* Mos protein (shown by 30K) that was expressed in *E. coli*²² were used as standards for quantitation. The 5S antibody (shown by IgG) used for immunoprecipitation also reacted with ^{125}I -labelled protein A used as a probe. *c*, *In vitro* kinase assay of pp39^{mos} in fully matured oocytes. Cytosol from 800 *in vitro*-matured oocytes was immunoprecipitated using 5S antibody in the presence (+) or absence (-) of a competing synthetic peptide and subjected to an *in vitro* kinase assay. Relative molecular mass markers (M_r) are shown.

METHODS. Ovaries were manually dissected into small pieces and incubated for 13 h in MBS containing collagenase (2 mg ml⁻¹) in the presence or absence of progesterone (5 $\mu\text{g ml}^{-1}$). Fully matured oocytes as well as immature (stage VI) oocytes were collected and subjected to cytosol preparation exactly as described by Shibuya and Masui²⁸. Typically, 1.2–1.5 ml of cytosol was recovered from 3,000 oocytes and to this was added 10 mM NaF, 10 mM MgSO₄, 6 mM ATP and 1 mM 2-mercaptoethanol at final concentrations as described²⁸. Immunoprecipitation of Mos protein from the cytosol was performed as follows. Cytosol was pre-absorbed with protein A-Sepharose (5 mg ml⁻¹), followed by the addition of the 5S monoclonal antibody (as ascites) (20 $\mu\text{l ml}^{-1}$) in the presence or absence of a competing synthetic peptide (10 $\mu\text{g ml}^{-1}$). After incubation at 2 °C for 10 h (for western blot analysis) or 3 h (for the *in vitro* kinase assay), the immune complexes were recovered by the addition of protein A-Sepharose (5 mg ml⁻¹); under these conditions, the recovery of Mos protein was more than 95%. For western blot analysis, the immune-complexes (with protein A-Sepharose) were washed with RIPA buffer (see the legend to Fig. 1), subjected to SDS-PAGE and transferred to a nylon membrane filter (GVHP, Millipore). The filter was coated with bovine serum albumin and incubated with 5S antibody diluted 100-fold with rinse buffer (10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA and 0.1% Triton X-100), washed with the rinse buffer and probed with ^{125}I -labelled protein A (0.5 $\mu\text{Ci ml}^{-1}$). Excess ^{125}I -protein A was washed off with the rinse buffer containing 1 M NaCl and the filter was autoradiographed. The *in vitro* kinase assay was performed using the immune-complexes (bound to protein A-Sepharose) described above according to the methods described previously^{48,49}. The immune complexes were washed with wash buffer⁴⁸ and subjected to SDS-PAGE analysis.

by Shibuya and Masui²⁸, in which packed oocytes are crushed by ultracentrifugation in an extraction buffer containing EGTA. Cytosol obtained in this way and subjected to immunoprecipitation and western blot analysis using 5S monoclonal antibody, clearly revealed pp39^{mos} polypeptide (Fig. 2b). This polypeptide of relative molecular mass 39,000 (39K) was not detected when western blot analysis was performed in the presence of a competing Mos C-terminal peptide (data not shown), nor was it detected in immature oocyte extracts (Fig. 2b). Using serial dilutions of *Escherichia coli*-produced Mos protein²² as a standard (Fig. 2b), we estimate that a single mature oocyte has ~200 pg of pp39^{mos} or 0.001% of total soluble protein, a value close to that of viral Mos in transformed cells (see above). Also, we found that >85% of the pp39^{mos} was in the cytosol extract indicating, as with viral Mos protein²⁹, a localization to this compartment (data not shown).

The Mos protein is believed to be a serine/threonine protein kinase^{30,31}, but in any systems including *Xenopus*, its kinase activity has never been detected, presumably because of its scarcity and fragility. To test for *in vitro*-Mos kinase activity, therefore, we prepared cytosol extracts as above from as many as one thousand *in vitro*-matured oocytes. The Mos protein was immunoprecipitated from the cytosol in extraction buffer using the 5S monoclonal antibody in the presence or absence of a competing Mos peptide. Kinase activity was assayed by adding [γ -³²P]ATP to the immunoprecipitates. In the absence of the competing peptide, a 39K polypeptide as well as abundant (55–58K) polypeptides were phosphorylated (Fig. 2c). After extensive washing of the phosphorylated immunoprecipitates with standard immunoprecipitation buffer containing ionic detergents, only the 39K protein was detected (data not shown), consistent with it being pp39^{mos}. In the presence of the competing peptide, no labelled proteins were detected, indicating that the 55–58 K polypeptides were co-precipitated with and phosphorylated by pp39^{mos} (Fig. 2c). Also, no phosphorylated proteins were detected when the cytosol of immature oocytes was

analysed (data not shown). We conclude that pp39^{mos} has autophosphorylating activity and can also phosphorylate co-precipitated proteins such as the 55–58 K products.

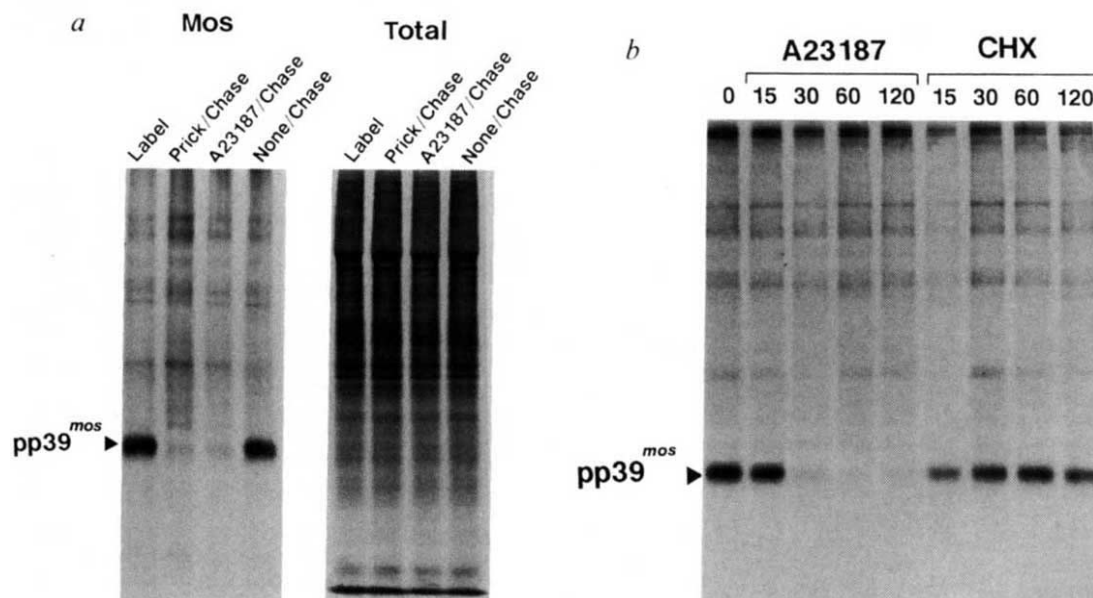
Destruction of pp39^{mos} on fertilization

These results show that the *in vitro*-matured oocytes or unfertilized eggs contain a store of stable and hyperphosphorylated pp39^{mos} associated with an intrinsic kinase activity. By contrast, it has been shown that synthesis of pp39^{mos} protein is not detectable after fertilization^{22,26}. To test whether pp39^{mos} persists after fertilization, we examined its stability after egg activation. The *in vitro* matured oocytes prelabelled with [³⁵S]methionine were activated either by pricking with a glass needle or treatment with calcium ionophore (A23187). After 1 h, egg extracts were immunoprecipitated using the 5S monoclonal antibody. These analyses revealed that the stable pp39^{mos} product disappeared completely after egg activation (Fig. 3a, left). We observed a similar disappearance of pp39^{mos} in inseminated eggs²⁶, indicating that this occurs during natural fertilization. By contrast, we did not observe appreciable degradation of pp39^{mos} without activation (Fig. 3a, left), nor did we observe any gross degradation of total proteins in eggs whether activated or not (right panel in Fig. 3a). These results indicate that pp39^{mos} is specifically degraded soon after egg activation.

To determine how fast the degradation of pp39^{mos} occurs after egg activation, *in vitro*-matured oocytes prelabelled with [³⁵S]methionine were either activated with calcium ionophore or treated with cycloheximide. They were then cultured and collected for the indicated times and subjected to immunoprecipitation analysis (Fig. 3b). Between 15 and 30 min after activation (a time ~10 min after the completion of meiosis^{6,32}), almost all of the pp39^{mos} disappeared (A23187 in Fig. 3b). By contrast, in the cycloheximide-treated oocytes, pp39^{mos} persisted for more than two hours, demonstrating that its natural decay is very slow in mature oocytes (CHX in Fig. 3b; see also Fig. 2a). We did not observe any appreciable

FIG. 3 Selective proteolysis of pp39^{mos} upon egg activation. *a*, Degradation of pp39^{mos} upon egg activation. *In vitro* matured oocytes prelabelled with [³⁵S]methionine (Label) were washed and chased for one hour without activation (None/Chase) or after activation by either pricking with a glass needle (Prick/Chase) or treatment with calcium ionophore (A23187/Chase). These oocytes were subjected to SDS-PAGE either directly (Total) or after immunoprecipitation with 5S monoclonal antibody (Mos). The very faint bands in activated eggs (Mos) that comigrate with pp39^{mos} are not Mos proteins, because they were not competed out by a competing Mos synthetic peptide (not shown). *b*, The time course of selective degradation of pp39^{mos} after egg activation. *In vitro* matured oocytes pre-labelled with [³⁵S]methionine were washed (time 0) and chased in the presence of either calcium ionophore (A23187) or cycloheximide (CHX) for the period indicated (in minutes) and then subjected to immunoprecipitation using the 5S antibody.

METHODS. Fully grown oocytes obtained as described in Fig. 1 were labelled continuously with [³⁵S]methionine (1 mCi ml⁻¹) for 13 h in MBS containing



progesterone (5 μ g ml⁻¹). After being washed with MBS, the labelled matured oocytes were either pricked with a glass needle or treated with calcium ionophore (A23187; 1 μ g ml⁻¹) for activation, or treated with cycloheximide (300 μ g ml⁻¹) without activation. In the cycloheximide-treated oocytes, protein synthesis was inhibited by more than 95% within 30 min after the treatment. The oocytes were cultured for the period specified and subjected to immunoprecipitation as described in Fig. 1.

degradation of total protein during the 2-h chase in the presence of cycloheximide (data not shown). These results clearly show that pp39^{mos} is rapidly and selectively destroyed soon after the completion of meiosis.

Involvement of calpain in pp39^{mos} proteolysis

Many events after fertilization or egg activation have been known to be triggered by a calcium ion influx³³. We suspected that this could induce activation of some specific protease that might be responsible for the hydrolysis of pp39^{mos}. Indeed, in preliminary experiments, loss of pp39^{mos} was prevented if oocytes matured *in vitro* were treated with the protease inhibitor leupeptin before egg activation (data not shown). To determine the protease involved in pp39^{mos} proteolysis, we established an *in vitro* assay system. A mixture of [³⁵S]methionine-prelabelled and unlabelled matured oocytes was centrifuged and cytosol extracts were prepared as described above (see Fig. 2 legend). When 5 mM calcium was added to the cytosol for *in vitro* activation²⁸, almost complete degradation of pp39^{mos} occurred after five hours at 2 °C, as determined by immunoprecipitation analyses (left panel in Fig. 4a). With the limitation that some degradation of total cellular polypeptides larger than 90K also occurs when calcium is added to this system (Fig. 4a, right), it was possible to study the specific degradation of pp39^{mos} *in vitro*. We tested the effect of various protease inhibitors (130 µM each) added to the cytosol before adding 5 mM calcium. Specific

inhibition of pp39^{mos} degradation was observed only with leupeptin, E-64 and antipain (Fig. 4b). As little as 10 µM E-64 also prevented degradation (data not shown). Leupeptin and antipain are known to inhibit certain kinds of both serine and cysteine proteases, whereas E-64 is a specific inhibitor of all cysteine proteases³⁴. The specificity of these inhibitors plus the requirement of calcium for pp39^{mos} proteolysis indicated that the responsible enzyme is a calcium-dependent cysteine protease.

Calpain or a calcium-activated neutral protease (CANP) has been shown to be well conserved in a variety of vertebrates^{35,36}, including amphibians (K. Tomita and K. Suzuki, personal communication). Calpain is a calcium-dependent cysteine protease and therefore a good candidate for effecting pp39^{mos} degradation. We first examined the existence of calpain in *Xenopus* oocytes. The cytosol of immature as well as *in vitro*-matured oocytes was immunoprecipitated and then analysed by western blot using an anti-chicken-calpain antibody³⁷. Only two polypeptides, of 80K and 27K, were specifically detected both in immature and mature oocytes (Fig. 5a). The relative molecular masses of these two polypeptides are very similar to the sizes (80K and 27–30K; refs 35, 36) of calpain subunits found in higher vertebrates. Thus, we strongly suspect that they represent subunits of the *Xenopus* calpain molecule.

The activity of calpain is inhibited by an endogenous proteinaceous inhibitor or calpastatin^{35,36,38}. Calpastatin is also well

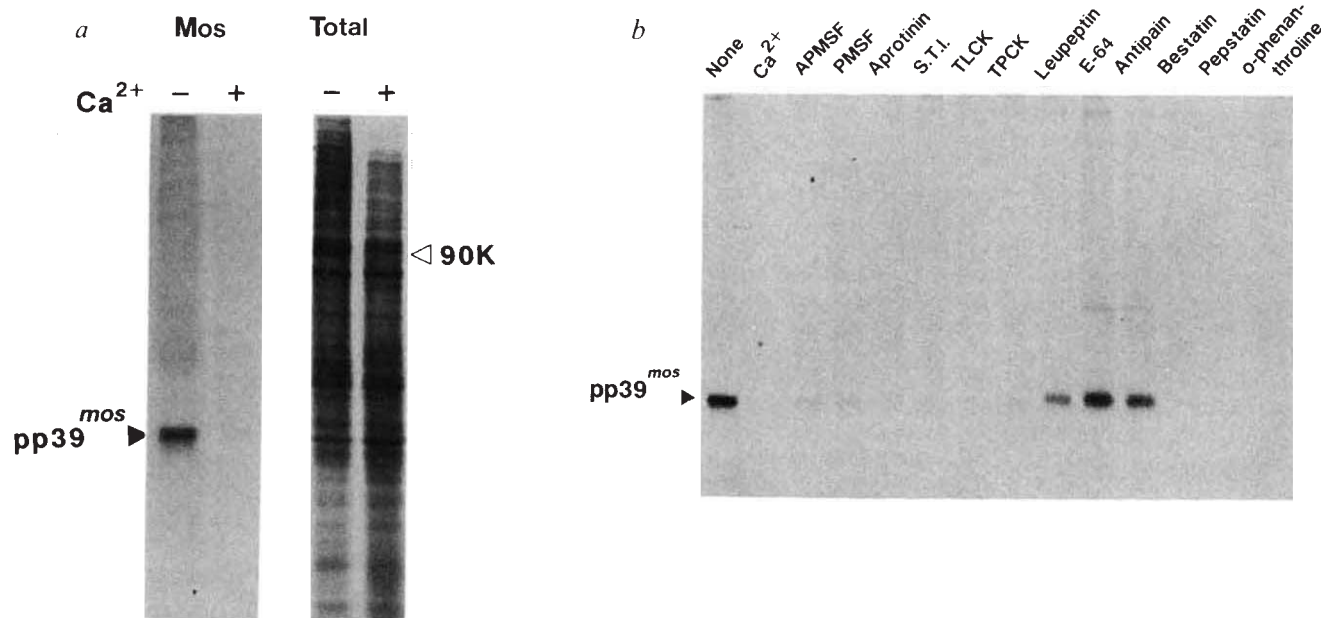


FIG. 4 *In vitro* analysis of proteolytic degradation of pp39^{mos}. a, Selective degradation of pp39^{mos} in egg cytosol by the addition of calcium. Cytosol obtained from a mixture of [³⁵S]methionine-labelled as well as unlabelled matured oocytes was incubated for 5 h at 2 °C in the presence (+) or absence (–) of calcium at 5 mM. The cytosol was subjected to SDS-PAGE either directly (Total) or after immunoprecipitation with the 5S antibody (Mos). In 'Total', only partial and non-specific degradation of polypeptides larger than 90 K is observed in the presence of calcium. b, Effect of various protease inhibitors on the *in vitro* degradation of pp39^{mos} by calcium. To the cytosol obtained as described in a, various kinds of protease inhibitors, as indicated, were added individually at a final concentration of 130 µM (unless specified below), followed by the addition of calcium and then incubated for 5 h at 2 °C. Cytosol without additions (None) or with the addition of calcium alone (Ca²⁺) served as control. All cytosol was subjected to SDS-PAGE after immunoprecipitation with the 5S antibody. Aprotinin, 50 U ml⁻¹ in KIE-unit; S.T.I., 10 µM; pepstatin, 13 µM. APMSF and TPCK (see figure) are primarily inhibitors of many serine proteases. Leupeptin and antipain inhibit certain kinds of both serine and cysteine proteases, while

E-64 is a specific inhibitor of all cysteine proteases. Bestatin, pepstatin and o-phenanthroline are inhibitors of aminopeptidases, acid proteases and metalloproteases, respectively. DFP (diisopropyl fluorophosphate), a specific inhibitor of all serine proteases, did not inhibit pp39^{mos} degradation at 130 µM (not shown). APMSF, (4-amidino-phenyl) methane-sulphonyl fluoride; PMSF, phenyl-methane-sulphonyl fluoride; S.T.I., soybean trypsin inhibitor; TLCK, L-1-chloro-3-4-tosylamido-7-amino-2-heptanone; TPCK, L-1-chloro-3-tosylamido-4-phenyl-2-butanone.

METHODS. Fifty fully grown oocytes were labelled with [³⁵S]methionine (1 mCi ml⁻¹) for 13 h in MBS containing progesterone (5 µg ml⁻¹) as described in Fig. 1 and mixed with about 3,000 unlabelled *in vitro*-matured oocytes that were prepared as described in the legend of Fig. 2. Cytosol was obtained from the mixed, *in vitro*-matured oocytes as described in Fig. 2, and to each aliquot (70 µl) of the cytosol was added each of the protease inhibitors, followed 10 min later by the addition of CaCl₂ to 5 mM and incubated for 5 h at 2 °C. The cytosol was mixed with an equal volume of 2 × RIPA buffer and analysed by immunoprecipitation using the 5S antibody.

conserved in vertebrate species and the calpastatin from one species can reversibly inhibit calpain of another species^{35,36}. Calpastatin has four repeated domains, each of which effectively inhibits calpain *in vitro*^{38,39}. To our cytosol protease assay system, we added the synthetic peptide (34 amino acids) that corresponds to the most effective inhibitor domain of the rabbit calpastatin⁴⁰. When 2 μ M calpastatin peptide was added to the cytosol before calcium addition, significant inhibition (~40%) of pp39^{mos} degradation occurred (Fig. 5b). At the higher concentrations of 10 μ M (Fig. 5b) and 50 μ M (data not shown), slightly greater inhibition (~60%) occurred. The reason for this incomplete inhibition even at higher concentrations is not clear (see discussion). The effect, however, of 2 μ M calpastatin peptide as well as 10 μ M E-64 (see above) strongly argues that calpain is the principal protease responsible for pp39^{mos} degradation.

Discussion

Although pp39^{mos} synthesis is induced before and is required for GVBD^{22,23}, it is present throughout maturation and is stably maintained in a hyperphosphorylated form in unfertilized eggs (Fig. 1). As recently reported for mouse Mos, this suggests that it also functions after GVBD²⁵. Interestingly, we have observed that pp39^{mos} synthesized 30 min before GVBD is not detected after a 2-h chase (Fig. 2a). This indicates that the turnover of pp39^{mos} is rapid during meiosis I, and possibly like MPF and cyclins^{2,11,41}, pp39^{mos} may be degraded at the completion of meiosis I, which occurs one hour after GVBD².

In the immune-complex pp39^{mos} kinase assay, we observed phosphorylation of co-precipitated 55–58 K polypeptides. These polypeptides were removed by extensive washing of the immune-complex with standard immunoprecipitation buffer, indicating that the association is weak. Using a cytosol extraction buffer containing no ionic detergent, we have also observed, in pp39^{mos} immunoprecipitates derived from ³²P-labelled matured oocytes, the co-precipitation of similarly sized polypeptides (data not shown), raising the possibility that *in vitro*-phosphorylated 55–58 K polypeptides may also be the *in vivo* substrates of pp39^{mos}. We do not know the nature of these polypeptides. It is interesting to note, however, that their molecular weights are similar to those of *Xenopus* cyclins^{42,43} (see accompanying paper²⁶).

We have demonstrated that pp39^{mos} stored in mature oocytes or unfertilized eggs is selectively degraded on egg activation (Fig. 3). Using an *in vitro* protease assay system (Fig. 4), we have shown that the degradation is inhibited by a synthetic peptide of calpastatin, an endogenous inhibitor of the calcium-dependent cysteine protease or calpain (Fig. 5b) and we indeed show that *Xenopus* oocytes contain calpain (Fig. 5a). Although as little as 2 μ M calpastatin peptide significantly inhibited pp39^{mos} degradation, we were not able to achieve complete inhibition even at much higher concentrations. Perhaps either the calpastatin peptides are rapidly degraded in our assay system or the heterologous calpain is in a conformation that prevents complete inhibition by rabbit calpastatin. It is also possible that as calpain is inactivated by calpastatin only after being activated by calcium⁴⁴, perhaps some calpain instantaneously degrades pp39^{mos} on calcium addition. Alternatively, there may be other calcium-dependent cysteine proteases that are also responsible for pp39^{mos} degradation. The specific inhibition of pp39^{mos} degradation by 2 μ M calpastatin, whereas other protease inhibitors fail to inhibit at levels of 130 μ M, leads us to conclude that endogenous calpain is responsible for the degradation. To our knowledge, this is the first demonstration of a specific protease that degrades a specific protein at egg activation or fertilization.

Although its physiological function is not known, calpain is ubiquitous and can degrade a variety of substrates *in vitro*^{35,36}. On egg activation, however, pp39^{mos} is the only detectable protein which is specifically degraded by calpain. Thus, although we cannot exclude that other proteins are also specifically

degraded, somehow calpain is regulated to specifically degrade pp39^{mos} at fertilization. Such specific degradation may be accomplished if, for example, calpain and pp39^{mos} are co-compartmentalized or physically associated in the egg. This co-compartmentalization or physical association could be partially disrupted *in vitro*, which could account for the small amount of degradation of total protein observed in our assay system (Fig. 4a). Similarly, calpain has recently been shown to function, and be compartmentalized, during mitotic metaphase-anaphase transitions⁴⁵.

In most animal species calcium influx occurs in eggs within minutes of fertilization^{33,46}. This transient increase in the level of intracellular free calcium triggers many events that follow fertilization³³. Evidently, the selective proteolysis of pp39^{mos} by calcium-activated calpain is one of those events. MPF activity and cyclins (components of MPF; refs 10–12) are also shown to be degraded by the increase in calcium on fertilization^{1,2,15,43}. Although its mechanism has not been elucidated, cyclin degradation certainly plays a crucial role in the inactivation of MPF^{11,15}. Although we suggest in the accompanying paper²⁶ that pp39^{mos} may function to stabilize MPF in the egg by protecting cyclins from proteolytic degradation, paradoxically, pp39^{mos} degradation occurs 15–30 min after egg activation (Fig. 3b). This is significantly later than cyclin degradation, which is reported to occur within 10 min of activation⁴³. This could mean either that the function (for example, kinase activity) of pp39^{mos}

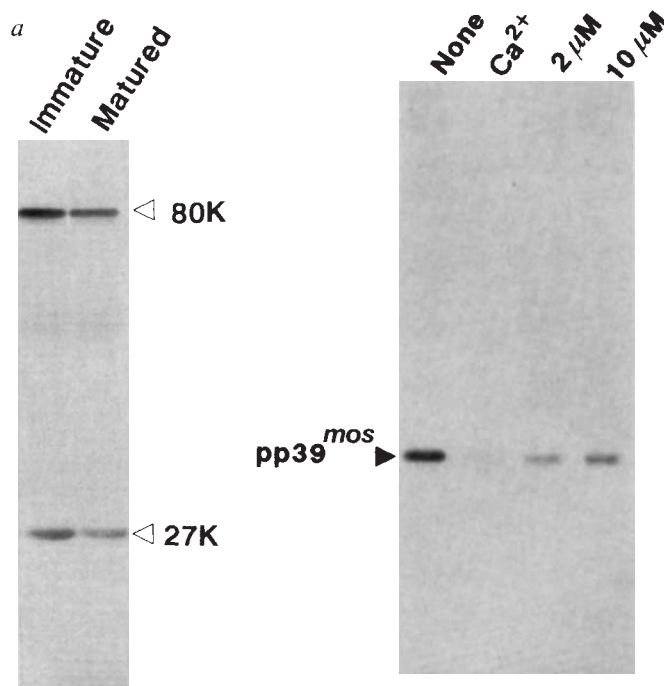
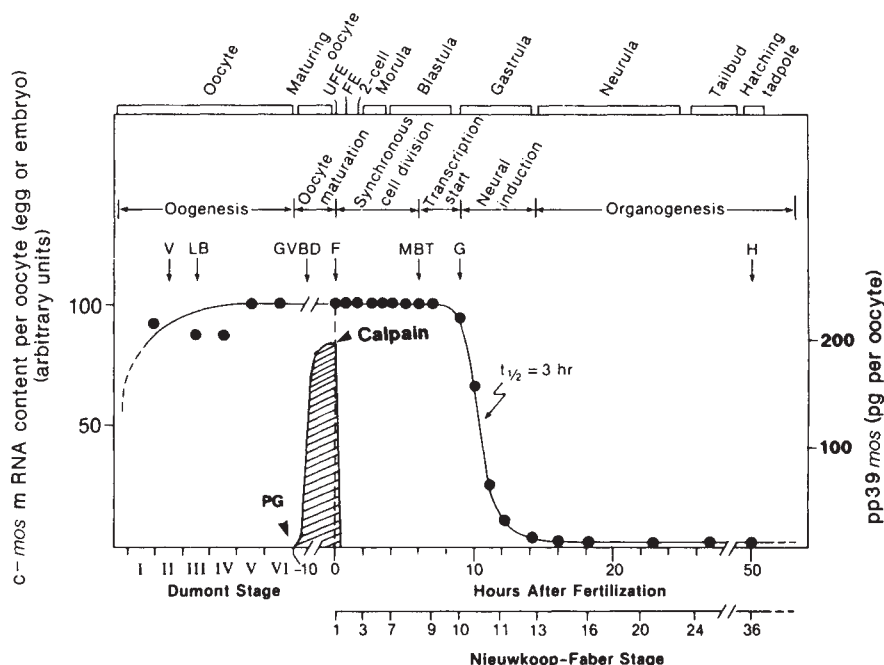


FIG. 5 Presence of calpain in *Xenopus* oocytes and inhibition of *in vitro* degradation of pp39^{mos} by calpastatin peptide. a, Detection of calpain in *Xenopus* immature and *in vitro*-matured oocytes. Cytosol was obtained from about 2,000 immature (stage VI) oocytes as well as *in vitro*-matured oocytes as described in the legend of Fig. 2. Cytosol equivalent to 70 oocytes was first immunoprecipitated using anti-chicken calpain antibody³⁷ and then subjected to western blot analysis using the same antibody under the same conditions as described in the legend of Fig. 2. b, Effect of calpastatin peptide on the degradation of pp39^{mos} *in vitro*. Cytosol was obtained as described in the legend of Fig. 4. To this cytosol (70 μ l) was added a synthetic peptide at 2 μ M and 10 μ M that corresponds to amino acids 184–217 of the rabbit calpastatin^{38,40}. After 10 min, 5 mM CaCl₂ was added to the cytosol, which was then incubated for 5 h at 2°C and analysed by immunoprecipitation using the 5S antibody, as described in Fig. 4.

FIG. 6 Expression patterns of *c-mos* RNA and Mos protein (pp39^{mos}) during early development of *Xenopus laevis*. The data for *c-mos* RNA (dots) were taken from ref. 22, those for Mos protein (hatched area) were from refs 22, 23, and 26 and this paper. The developmental stages for oogenesis and embryogenesis are according to Dumont⁴⁷ and Nieuwkoop and Faber⁵⁰, respectively. (See text for details.) F, fertilization; FE, fertilized egg; G, gastrulation; GVBD, germinal vesicle breakdown; H, hatching; LB, lampbrush stage; MBT, mid-blastula transition; UFE, unfertilized egg; V, start of vitellogenesis.



is somehow inactivated before it is degraded by calpain, or that there are other mechanisms by which it stabilizes MPF.

We are now able to depict the entire course of synthesis, accumulation and degradation of pp39^{mos} during *Xenopus* early development (Fig. 6). The *c-mos* RNA is transcribed and stored as a maternal mRNA in oocytes and persists throughout early embryogenesis until gastrulation. Despite this persistence, detectable translation of the *c-mos* maternal RNA occurs only during progesterone-induced oocyte maturation. During this period, pp39^{mos} accumulates (it may be degraded once at the end of meiosis I, when the MPF activity also falls²; Fig. 2a) and is phosphorylated. In the unfertilized egg, pp39^{mos} is very

stable, is hyperphosphorylated and has kinase activity. At fertilization or soon after the completion of meiosis, stored pp39^{mos} undergoes rapid and selective degradation by endogenous calpain which is activated by the increase in intracellular free calcium. These unique patterns of pp39^{mos} expression strongly suggest that its function during early development is restricted to meiosis. These expression patterns are strikingly similar to those of cytosolic factor (CSF)³ and indeed we show in the accompanying paper²⁶ that pp39^{mos} is CSF. □

Note added in proof: We find that the degradation of cyclins is not inhibited by calpastatin in our protease system.

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The *c-mos* proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs

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The *c-mos* proto-oncogene product, pp39^{mos}, is present in unfertilized *Xenopus* eggs, and disappears on fertilization. Microinjection of synthetic *mos* RNA into two-cell embryos induces cleavage arrest at metaphase. By contrast, egg cytosol extracts, when immunodepleted of endogenous pp39^{mos}, lose their cleavage-arresting activity in injected embryos. These results demonstrate that Mos protein is the cytostatic factor CSF, long known as an endogenous meiotic inhibitor in vertebrate eggs.

IN most animal species, fully grown oocytes are naturally arrested at the first meiotic prophase, or prophase I¹. This arrest is usually released by hormones, and meiosis resumes followed by the appearance of a cytoplasmic activity, called maturation promoting factor (MPF), that causes germinal vesicle breakdown (GVBD) and chromosome condensation¹⁻³. It is now known that MPF is ubiquitous in eukaryotes⁴, promotes transition from G2 into meiosis and mitosis^{5,6} and contains two components, p34^{cdc2} kinase and cyclin⁷⁻¹².

In vertebrates, meiosis is arrested again at the second meiotic metaphase or metaphase II before fertilization¹³. This meiotic arrest is released by fertilization or egg activation to complete meiosis^{1,13}. It has been postulated for over half a century that fertilization acts to remove a meiotic inhibitor present in the unfertilized egg¹⁴⁻¹⁶. In fact, about two decades ago Masui and Markert², using the amphibian system, demonstrated that microinjection of unfertilized egg cytoplasm into blastomeres of two-cell embryos can induce cleavage arrest at metaphase. This cytoplasmic activity has been ascribed to a factor, designated cytostatic factor (CSF)². The CSF activity appears as GVBD occurs during oocyte maturation, it remains high in the unfertilized egg and disappears soon after fertilization, when the second meiosis is completed^{2,17,18}. The disappearance of CSF activity at fertilization is believed to be caused by a transient increase in cytoplasmic free calcium on fertilization¹ and is indeed calcium-sensitive *in vitro*^{17,19}. These properties of CSF satisfy the criteria for the presumed endogenous meiotic inhibitor^{17,20}. Like MPF, CSF seems to be ubiquitous^{13,20} (at least in vertebrates) and most probably functions to stabilize MPF activity in the unfertilized egg²¹⁻²³. Unlike MPF, however, little is known about the chemical nature or molecular composition of CSF¹⁹.

The *c-mos* proto-oncogene, encoding a putative serine/threonine protein kinase²⁴, is expressed at high levels in the germ cells of vertebrates²⁵⁻²⁸. Recent studies indicate that synthesis of the endogenous *Xenopus* Mos protein, pp39^{mos}, is uniquely stimulated during progesterone-induced oocyte maturation and is required for both activation of MPF and GVBD^{29,30}. Expression of the Mos protein is also necessary for completion of mouse oocyte maturation³¹.

In the accompanying paper³², we show that the *Xenopus* pp39^{mos} is continuously synthesized during maturation and accumulates in mature oocytes or unfertilized eggs as a hyperphosphorylated form that exhibits protein kinase activity. We also demonstrate³² that the accumulated pp39^{mos} is selectively

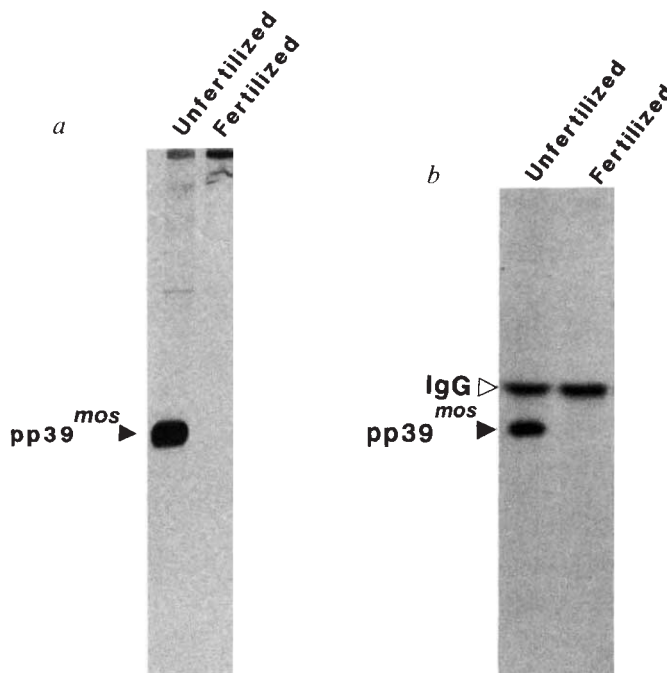


FIG. 1 Absence of *c-mos* product (pp39^{mos}) in *Xenopus* fertilized eggs. *a*, Immunoprecipitation analysis of pp39^{mos} in unfertilized and fertilized eggs labelled with [³⁵S] methionine. *b*, Western blot analysis of pp39^{mos} in unfertilized and fertilized eggs. The 5S monoclonal antibody (shown by IgG) used for immunoprecipitation is also detected.

METHODS *a*, Ten fully (stage VI; ref. 47) oocytes were obtained²⁹ and labelled with [³⁵S]methionine (1 mCi ml⁻¹) for 13 h at 20 °C in modified Barth solution (MBS) containing progesterone (5 µg ml⁻¹). These *in vitro* matured oocytes served as unfertilized eggs. Ten ovulated eggs⁴⁸ were fertilized *in vitro* and dejellied with cysteine²¹. Fifteen minutes after fertilization, the eggs were injected with [³⁵S]methionine (5 µCi per egg) in 0.3 × MBS containing 5% Ficoll and cultured at 23 °C in the same solution for 2 h, when the fertilized eggs reached the four-cell stage. Two unfertilized eggs and ten fertilized eggs were subjected to immunoprecipitation using 5S monoclonal antibody³⁰ as described in the legend of Fig. 1 in the accompanying paper³². *b*, Fully grown oocytes were obtained²⁹ and induced to mature with progesterone (5 µg ml⁻¹) for 13 h at 20 °C in MBS and served as unfertilized eggs. Fertilized eggs were collected one hour after *in vitro* fertilization of ovulated eggs²¹. Cytosols were prepared from unfertilized as well as fertilized eggs as described (ref. 19 and see also accompanying paper³²). Cytosol equivalent to 600 eggs was subjected to immunoprecipitation and then western blot analysis using 5S monoclonal antibody as described in the legend of Fig. 2 in the accompanying paper³².

destroyed by a calcium-dependent calpain protease on egg activation. Thus, the properties of pp39^{mos} show striking similarities to those of CSF described above. In this report, we show that microinjection of synthetic *c-mos* RNA into *Xenopus* two-cell embryos induces cleavage arrest at metaphase and that this arrest is associated with high MPF activity. Also, we demonstrate that unfertilized egg cytosol, if immunodepleted of endogenous pp39^{mos} and then injected into two-cell embryos, fails to induce cleavage arrest. Collectively, our results firmly establish that Mos protein is either CSF or a catalytic component of it.

Fertilized eggs lack pp39^{mos}

Previously, we have shown that pp39^{mos} synthesis is not detectable in cleavage and blastula embryos of *Xenopus laevis*, although *c-mos* maternal mRNA is²⁹. We used the pp39^{mos}-specific monoclonal antibody (termed 5S; ref. 30) for immunoprecipitation analysis of zygotes labelled with [³⁵S]methionine 15 min to 135 min after fertilization, during the 1 to 4-cell stages. Figure 1a shows that pp39^{mos} is not detected in these early embryos, whereas the product is easily detected in unfertilized eggs labelled during the course of maturation *in vitro*. In conjunction with previous results²⁹, these results clearly indicate that pp39^{mos} is not synthesized after fertilization, despite the persistence of *c-mos* maternal messenger RNA²⁹.

If pp39^{mos} is degraded in unfertilized eggs during fertilization (accompanying paper³²) and translation of *c-mos* maternal mRNA is blocked after fertilization (Fig. 1a), then we should not observe pp39^{mos} in eggs after fertilization. To test this,

unfertilized eggs and eggs one hour after fertilization were subjected to western blot analysis using the 5S monoclonal antibody. As expected, pp39^{mos} is not detected in fertilized eggs, whereas it is readily detected in unfertilized eggs (Fig. 1b).

C-mos RNA induces cleavage arrest

The destruction of pre-existing pp39^{mos} at fertilization and the simultaneous block of *c-mos* maternal mRNA translation could mean either that pp39^{mos} is not required after fertilization or that it must be removed to allow normal post-fertilization development to occur. To test either consideration, we microinjected synthetic *c-mos* RNA³⁰ as well as many other *mos*-related and unrelated RNA species into one blastomere of a two-cell embryo (Table 1). The 5'-capped normal *c-mos* RNA (CN-*mos* RNA) directs the synthesis of pp39^{mos} in injected embryos (data not shown). When 2–20 ng (per embryo) of this RNA was injected into one of the blastomeres of two-cell embryos, 100% of the injected blastomeres ceased cleavage within 30 to 90 min after injection (Table 1) and this resulted in hemiblastulation (Fig. 2a, right). Only the injected blastomeres showed cleavage arrest at two-cell, four-cell or eight-cell stages. These experiments also demonstrate that as we increase the amount of injected CN-*mos* RNA, cleavage arrest occurred earlier (Table 1). When a lesser amount of CN-*mos* RNA was injected (1 ng), the majority (96%) of the injected blastomeres still showed cleavage arrest, but this occurred at significantly later stages (16 to 32-cell and 64 to 256-cell stages, Table 1). At the lowest dose examined (0.2 ng), many embryos developed normally but a

FIG. 2 Morphology of embryos and chromosomes as well as MPF activity in *mos* RNA-arrested embryos. a, Animal view of embryos injected with either 5'-capped truncated (CT-) or 5'-capped normal (CN-) *mos* RNA. Synthetic *mos* RNA was injected into one blastomere (right half) of a two-cell embryo. Cleavage arrest is observed only when injected with CN-*mos* RNA (right). b, Staining of chromosomes in uninjected control and CN-*mos* RNA-injected blastomeres with the DNA-specific dye. Chromosomes in CN-*mos* RNA-injected blastomeres are condensed and on a metaphase plate (CN-*mos*), whereas those in most of the uninjected control blastomeres are decondensed (control). c, MPF activity in uninjected control, CN-*mos* RNA-injected embryos and *in vitro* matured oocytes. Crude MPF preparations obtained from embryos and oocytes were serially diluted as indicated and injected into cycloheximide-treated fully grown oocytes. MPF activity is represented by bars as % GVBD of the recipient oocytes. Numbers of recipient oocytes and those undergoing GVBD are also indicated. METHODS a, CT-*mos* RNA or CN-*mos* RNA (5 ng of each) was injected into one blastomere of a two-cell embryo as described in Table 1. Injected embryos were cultured for 2 h at 23 °C in 0.3 × MBS containing 5% Ficoll and photographed. b, CN-*mos* RNA (5 ng) was injected into one blastomere of a two-cell embryo and cultured for 3 h as described above. Blastomeres of uninjected control and injected embryos were then fixed, stained with bisbenzimidazole H 33258 (Calbiochem.), squashed and photographed as described²¹. c, CN-*mos* RNA (5 ng) was injected into both blastomeres of each two-cell embryo, which resulted in cleavage arrest at the four-cell or eight-cell stage. Twenty embryos 3 h after the injection, uninjected control embryos, or *in vitro* matured oocytes were homogenized in 30 µl of a buffer containing 80 mM β-glycylphosphate, 15 mM EGTA and 10 mM MgCl₂ (pH 7.3) and crude MPF preparations were obtained as described³⁰. After serial dilutions of the MPF preparation with the above buffer, 60 nl of each MPF preparation was injected into fully grown oocytes pre-treated with cycloheximide (50 µg ml⁻¹) for 1 h. The injected oocytes were cultured for 5 h in MBS in the presence of 50 µg ml⁻¹ cycloheximide and examined for GVBD as described previously²⁹.

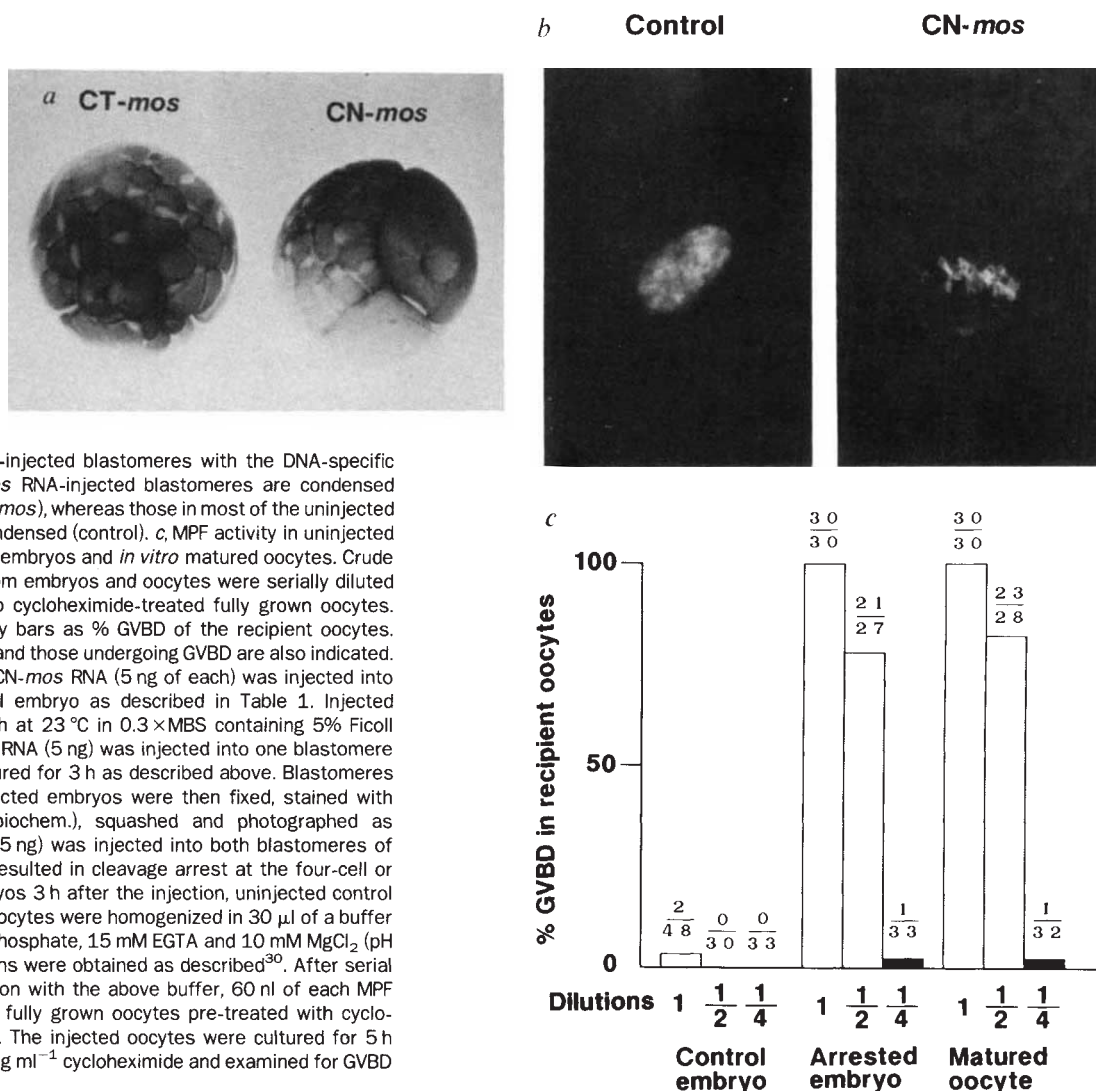


TABLE 1 Cleavage arrest induced by injected RNA

RNA species	Amount of RNA injected (ng per embryo)	Total no. of recipients	% Recipients arrested at stage						Total % arrested
			2-cell	4-cell	8-cell	16/32-cell	64/256-cell	mid- to late-blastula	
CN- <i>mos</i>	20	36	50	28	22	0	0	0	100
	5	115	24	65	11	0	0	0	100
	2	24	8	25	42	25	0	0	100
	1	50	0	0	0	56	40	0	96
	0.2	51	0	0	0	0	0	27	27
CN- <i>mos</i> + sense	5	26	19	50	31	0	0	0	100
CN- <i>mos</i> + antisense	5	28	0	0	0	0	0	0	0
UN- <i>mos</i>	5	21	0	0	0	0	0	0	0
CT- <i>mos</i>	20	35	0	0	0	0	0	0	0*
	5	63	0	0	0	0	0	0	0
	2	28	0	0	0	0	0	0	0
UT- <i>mos</i>	5	21	0	0	0	0	0	0	0
TMV	5	30	0	0	0	0	0	0	0
globin	5	27	0	0	0	0	0	0	0
tRNA	5	30	0	0	0	0	0	0	0
<i>Xenopus</i> oocyte	5	30	0	0	0	0	0	0	0

Ovulated eggs were obtained⁴⁸ and fertilized *in vitro*²¹. The fertilized eggs were dejellied with cysteine and cultured in 0.3 × modified Bartl solution (MBS) containing 5% Ficoll (Pharmacia) at 23 °C (ref. 29). Just before the completion of the first cleavage (or 90–100 min after fertilization), one blastomere of each two-cell embryo received in its pigmented (or animal) half an injection of 30 nl of a solution containing RNA as described²⁹. The injected embryos were cultured under the same conditions as above until the mid-blastula stage, when they were transferred into 0.1 × MBS without Ficoll and cultured further at 23 °C. When the blastomeres were successfully arrested by injection, they showed no signs of degeneration or deterioration for at least 7–8 h but eventually underwent cytolysis at about the mid- to late-gastrula stages (stages 11–12; ref. 49) or 10–12 h after the injection. Some injected blastomeres ceased cleavage with irregular pigment patterns and these embryos were omitted from the tabulated data. The RNAs used for injection were 5'-capped or uncapped normal *mos* RNA (CN-*mos* and UN-*mos*, respectively; see ref. 30 for the preparation of these RNAs), 5'-capped or uncapped truncated *mos* RNA (CT-*mos* and UT-*mos*, respectively; see below), tobacco mosaic virus (TMV) mRNA (Amersham), rabbit globin mRNA (BRL), yeast tRNA (Sigma) and *Xenopus* oocyte (stage VI; ref. 47) total RNA. In the experiments indicated, the CN-*mos* RNA (5 ng) was preannealed before injection with *Xenopus* c-*mos*-specific antisense or sense oligodeoxyribonucleotide (3 ng) (G⁻ and G⁺ oligonucleotides, respectively, in ref. 29) as described previously⁵⁰. CT-*mos* and UT-*mos* RNAs were prepared as follows. A *Ban*I-*Eco*RI fragment (845 base pairs (bp)) was isolated from a pSP64-*mos* construct previously described³⁰ and ligated to a synthetic double-stranded *Sac*I-*Ban*I oligonucleotide (CACCATGGAATACACCG), where the underlined ATG, which was originally a second ATG codon of the *Xenopus* c-*mos* coding region²⁹, served as an initiator methionine codon for the truncated *mos* RNA. This ligated fragment was inserted between *Sac*I and *Eco*RI sites of the pSP64 plasmid⁵¹ and transcribed *in vitro* with (for CT-*mos* RNA) or without (for UT-*mos* RNA) a 5'-cap structure as described³⁰. These RNAs, when translated, directed the synthesis of a truncated c-*mos* polypeptide consisting of the C-terminal 218 amino acids of the normal Mos protein²⁹. For each RNA species listed, at least two independent experiments were performed.

* At a dose of 20 ng of CT-*mos* RNA, all of the injected embryos developed normally at least up to the mid-blastula stage (stage 8 $\frac{1}{2}$; ref. 49), but most of them ceased development and underwent cytolysis at the initial gastrula stage, presumably because of the toxic effect of the high dose of RNA injected⁵².

significant proportion (~30%) still underwent cleavage arrest at very late stages (mid- to late-blastula stages, Table 1). Strikingly, CN-*mos* RNA (5 ng) pre-annealed before injection with *mos*-specific antisense (but not sense) oligodeoxyribonucleotide (G⁻; ref. 29), did not induce cleavage arrest (Table 1). Also, 5 ng of the uncapped form of normal *mos* RNA (UN-*mos* RNA, Table 1) failed to induce cleavage arrest. UN-*mos* RNA is unstable and not translated in injected embryos (data not shown). These results demonstrate that exogenously injected *mos* RNA induces cleavage arrest when translated in a two-cell embryo. The delayed cleavage arrest observed at the lower doses of CN-*mos* RNA may indicate that cleavage arrest requires accumulation of a critical level of pp39^{mos}.

We also injected truncated forms of c-*mos* RNA into blastomeres of two-cell embryos; this RNA synthesizes a short Mos protein that lacks the ATP-binding domain that is essential for Mos kinase activity³³ (data not shown). In sharp contrast with CN-*mos* RNA, neither uncapped nor capped forms of this truncated *mos* RNA (UT- and CT-*mos* RNA, respectively, Table 1) induced cleavage arrest at the doses tested (2–20 ng per embryo) (Fig. 2a, left). As further controls, we also injected 5 ng each of tobacco mosaic virus (TMV) mRNA, globin mRNA, yeast transfer RNA or *Xenopus* oocyte total RNA into two-cell embryos. Again, none of these RNAs caused cleavage arrest and the embryos developed quite normally.

The above results clearly show that cleavage arrest in injected embryos only occurs when *mos* RNA is translated into pp39^{mos}. We observed that cleavage-arrested embryos 3 h after injection with 2 ng of CN-*mos* RNA, contain approximately the same amount of pp39^{mos} as do unfertilized eggs (data not shown). It follows, therefore, that pp39^{mos} present in the unfertilized

egg must be removed after fertilization for normal cleavage to occur.

Properties of cleavage-arrested blastomeres

In the above experiments, cleavage-arrested blastomeres showed no signs of degeneration for at least 7–8 h and their surfaces remained smooth. We determined whether the *mos* RNA-injected blastomeres were arrested at a specific stage of the cell cycle. Cleavage-arrested blastomeres, one to five hours after arrest, were stained with fluorescent DNA-binding dye, squashed and observed with fluorescence microscopy. In all of more than 20 specimens, we observed that each arrested blastomere possessed a single nucleus, indicating that the cleavage arrest was due to failure in mitosis and not in cytokinesis. Also, in all the arrested blastomeres, the nucleus lacked a nuclear membrane and the chromosomes were fully condensed. Often, the condensed chromosomes were on the so-called metaphase plate (Fig. 2b, right). By contrast, most of the nuclei of uninjected control blastomeres had nuclear membranes and uncondensed chromosomes characteristic of interphase (left panel in Fig. 2b), with only ~10% (on average) having condensed metaphase chromosomes. These results clearly show that blastomeres injected with *mos* RNA are specifically arrested at metaphase.

Both meiotic and mitotic metaphases are associated with high MPF activity^{5,6,21}. We determined whether metaphase-arrested blastomeres also have high MPF activity. In these experiments, 5 ng of CN-*mos* RNA was injected into both blastomeres of a two-cell embryo, which allowed us to measure MPF activity from whole embryos arrested at either the four-cell or eight-cell stages. MPF activity was assayed by injection of serial dilutions of the crude MPF extracts into cycloheximide-treated fully

grown oocytes^{30,34}. Undiluted and twofold-diluted MPF extracts from the *mos* arrested embryos induced 100% and 78% GVBD, respectively, in recipient oocytes, whereas the fourfold dilution induced only 3% GVBD (Fig. 2c). By contrast, undiluted MPF prepared from uninjected control embryos induced only 4% GVBD and presumably reflected the low numbers of metaphase cells in normally cleaving blastomeres. We also show that the MPF activity of *in vitro*-matured eggs is very similar to that of the cleavage-arrested embryos (Fig. 2c).

Identification of pp39^{mos} as CSF

All the properties and activities of pp39^{mos} described above show striking similarities with CSF, the metaphase II-arresting activity found in amphibian unfertilized eggs. This prompted us to examine a possible relationship between pp39^{mos} and CSF by neutralization and immunodepletion experiments using Mos-specific antibodies (Table 2).

CSF activity is measured by the ability of unfertilized egg cytoplasm or cytosol to induce cleavage arrest in one injected blastomere of a two-cell embryo^{2,17}. We prepared cytosols from *in vitro* matured eggs according to the method of Shibuya and Masui¹⁹ (see also the accompanying paper³²). Fresh cytosol was treated with Mos-specific antibodies and then injected into one blastomere of a two-cell embryo. Untreated control cytosol exhibited a strong CSF activity, inducing very early cleavage arrest (at the 1 to 4-cell stage) in 96% of the recipient blastomeres (Table 2). Cytosol treated with the 5S monoclonal antibody also induced cleavage arrest, although the effect was somewhat delayed. When cytosol was treated with the B-Mos polyclonal antiserum that was raised against a bacterially-expressed Mos protein²⁹, however, CSF activity was markedly reduced and virtually no cleavage arrest was observed (Table 2). Normal rabbit serum had no effect on CSF activity. Thus, the B-Mos antiserum can neutralize CSF activity, indicating that pp39^{mos} is required for CSF activity. The lack of CSF neutralization by the 5S monoclonal antibody is presumably due to its inability to suppress pp39^{mos} function. We have demonstrated, for example, that pp39^{mos} kinase activity is not affected by the 5S monoclonal antibody (accompanying paper³²).

As further and direct evidence that pp39^{mos} is required for CSF activity, we removed it from cytosol by absorption to Mos-specific antibodies bound to protein A-Sepharose. The Mos-free cytosol was then assayed for CSF activity as above. Mock-treated control cytosol had a strong CSF activity and 97% cleavage arrest was observed at very early stages (Fig. 3, left,

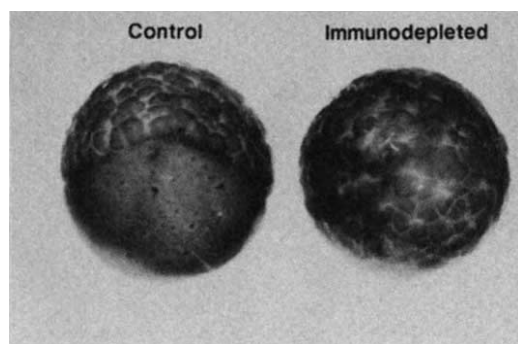


FIG. 3 Loss of CSF activity in egg cytosols immunodepleted of the endogenous Mos protein. Cytosol was obtained from *in vitro*-matured oocytes and either mock-immunodepleted or immunodepleted of the endogenous Mos protein using the 5S monoclonal antibody as described in the legend to Table 2. One blastomere (lower half in the figure) of a two-cell embryo was injected with 30 nl of cytosol either mock-immunodepleted (Control) or immunodepleted with the 5S antibody (Immunodepleted). The injected embryos were cultured for 3.5 h before photography.

and Table 2). By contrast, cytosol treated with B-Mos antiserum was markedly reduced in CSF activity (14% cleavage arrest occurred only at later stages; Table 2), a result similar to that observed with B-Mos antiserum in the neutralization experiment. Additionally, the 5S monoclonal antibody, which had little neutralization activity (Table 2), almost completely depleted the CSF activity (Fig. 3, right) with only 5% cleavage arrest (Table 2). Importantly, this immunodepletion by the 5S monoclonal antibody was abolished in the presence of a competing Mos C-terminal peptide³² (Table 2), establishing that the loss of CSF activity was due to a specific depletion of pp39^{mos} from the cytosol. We also used another Mos-specific polyclonal peptide antiserum, C232, which recognizes a different C-terminal region of pp39^{mos} (ref. 29). Again, CSF activity was greatly reduced from the cytosol (18% cleavage arrest, Table 2). Normal rabbit serum (as control for B-Mos and C232 antisera) or mouse IgG (as control for the 5S monoclonal antibody) did not diminish CSF activity at all (Table 2). These results

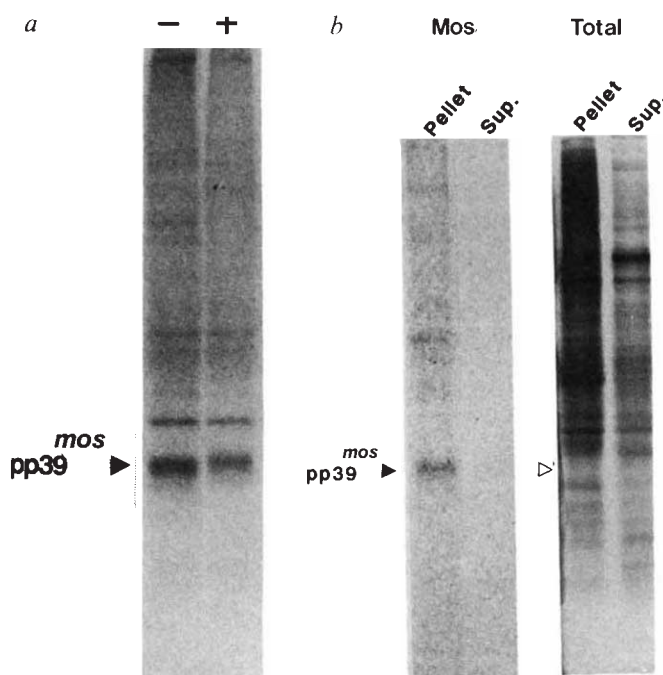


FIG. 4 Biochemical properties of pp39^{mos} in egg cytosols. *a*, Difference in the electrophoretic mobility of pp39^{mos} in cytosols that were stored for 24 h at 2 °C with (+) and without (-) the addition of 6 mM ATP and 10 mM NaF. Immunoprecipitation was performed with the 5S monoclonal antibody. *b*, Sedimentation of pp39^{mos} from egg cytosols. Pellet and supernatant (Sup.) fractions from cytosol centrifuged at 150,000g for 6 h were analysed by polyacrylamide gel electrophoresis either directly (Total) or after immunoprecipitation with the 5S antibody (Mos). The open triangle in Total indicates the apparent position of pp39^{mos}.

METHODS. Fifty fully grown oocytes were metabolically labelled for 13 h with [³⁵S]methionine (1 mCi ml⁻¹) in MBS containing progesterone (5 µg ml⁻¹). The labelled *in vitro* matured oocytes were mixed with ~3,000 unlabelled oocytes that were also induced to mature by progesterone. Cytosols were obtained from the mixed matured oocytes as described in the legend of Fig. 2 in the accompanying paper³². *a*, An aliquot (100 µl) of the cytosol prepared as described above was incubated at 2 °C for 24 h in the presence or absence of 6 mM ATP and 10 mM NaF (as well as 10 mM MgSO₄ and 1 mM 2-mercaptoethanol) as described¹⁹, mixed with an equal volume of 2 × RIPA buffer (see ref. 32) and subjected to immunoprecipitation with the 5S monoclonal antibody. *b*, The cytosol prepared as described above was subjected to a second centrifugation at 150,000g for 6 h at 2 °C as described²⁰. The supernatant was mixed with an equal volume of 2 × RIPA buffer, and the pellet was dissolved in 2 volumes of 1 × RIPA buffer. A portion (200 µl) from each fraction was analysed by immunoprecipitation with 5S monoclonal antibody, whereas another portion (10 µl) was electrophoresed directly on a polyacrylamide gel.

TABLE 2 Effect of Mos-specific antibodies on CSF activity

Antibodies	Total no. of recipients	1-cell*	% recipients arrested at stage				Total % arrested
			2-cell	4-cell	8-cell	>8-cell	
Neutralization							
None	27	8	67	22	0	0	97
B-Mos	51	0	4	0	2	0	6
5S	35	0	23	49	22	3	97
Normal rabbit serum	28	7	57	29	4	0	97
Immunodepletion							
None	70	6	63	28	0	0	97
None + Ca ²⁺	30	0	0	0	0	7	7
B-Mos	43	0	0	0	9	5	14
5S	66	0	0	0	2	3	5
5S + peptide	46	2	67	24	0	0	93
C232	48	0	0	4	8	6	18
Mouse IgG	51	4	74	10	8	0	96
Normal rabbit serum	54	2	62	20	5	4	93

Ovaries were manually dissected, cut into small pieces and incubated for 13 h at 20 °C in MBS containing progesterone (5 µg ml⁻¹) and collagenase (2 mg ml⁻¹). Fully matured oocytes were collected and subjected to cytosol preparation according to Shibuya and Masui¹⁹ (see also accompanying paper³²). After addition of 6 mM ATP, 10 mM NaF, 10 mM MgSO₄ and 1 mM 2-mercaptoethanol¹⁹, the fresh cytosols were subjected to neutralization as well as immunodepletion using the antibodies as indicated. In neutralization experiments 4 µl 5S monoclonal antibody (as ascites)³⁰, B-Mos antiserum²⁹ or normal rabbit serum, was added to 100 µl cytosol and this was then incubated for 30 min at 2 °C. In immunodepletion experiments, the 5S monoclonal antibody (5 µl of ascites containing 3 mg ml⁻¹ IgG), B-Mos polyclonal antiserum (15 µl), C232 polyclonal antiserum (15 µl)²⁹, mouse IgG (15 µl at 2 mg ml⁻¹) or normal rabbit serum (15 µl) was first adsorbed to 3 mg of protein A-Sepharose (Pharmacia). The antibody-protein A-Sepharose complex was washed with a cytosol extraction buffer¹⁹, added to 100 µl fresh cytosol and incubated for 4 h at 2 °C with continuous rotation. After the incubation, the reaction mixture was centrifuged briefly and a supernatant obtained as an immunodepleted cytosol. Under the present conditions, Mos-specific antibodies depleted more than 85% of Mos protein from the cytosol, the 5S monoclonal antibody being the most effective (more than 95% depletion; data not shown). In the experiment indicated, 15 µg synthetic Mos C-terminal peptide (see accompanying paper³²) was incubated in both the adsorption and immunodepletion steps for competition. Also as controls, mock-immunodepleted cytosols with (None + Ca²⁺) or without (None) the addition of CaCl₂ (5 mM) were prepared. Neutralized or immunodepleted cytosol (30 nl) was injected into one blastomere of each two-cell embryo and the injected embryos were cultured as described in the legend to Table 1. Embryos arrested with irregular pigment patterns were discarded. For each antibody in both neutralization and immunodepletion experiments, at least three independent experiments were performed.

* Arrest of both injected and uninjected blastomeres with occasional reversion of the first cleavage furrow.

clearly demonstrate that removal of pp39^{mos} protein from egg cytosol results in the specific loss of CSF activity and argue strongly that pp39^{mos} is either CSF itself or an essential component of its activity.

Properties of CSF and pp39^{mos}

Several biochemical properties of CSF have been characterized. Thus, CSF activity is sensitive to calcium^{17,19}, it can be stabilized by ATP and NaF¹⁹, and CSF sediments at 150,000 g after 6 h²⁰. If pp39^{mos} is CSF or part of it, then we would expect that its properties would be identical to those of CSF and would possibly contribute to the molecular basis of these. As previously reported¹⁹, the addition of 5 mM calcium to fresh cytosol inactivates CSF activity (Table 2). As shown in the accompanying paper³², pp39^{mos} is selectively degraded by calpain, a calcium-dependent protease. Thus, the inactivation of CSF activity by calcium could be due principally to the proteolytic degradation of CSF and is consistent with the observation that CSF activity never reappears after its inactivation^{2,18}.

Normally, CSF activity in cytosol lasts no more than 24 h at 2 °C (ref. 19 and our unpublished data), but the activity can be stabilized for up to a week by the addition of ATP and NaF¹⁹. Our experiments showed that without ATP and NaF, about two-thirds of the pp39^{mos} in the CSF cytosol is degraded after 24 h (data not shown). Thus, the loss of CSF activity during storage parallels the loss of pp39^{mos}. Although the quantity of pp39^{mos} is the same in cytosol stored for 24 h in the presence or absence of ATP and NaF, the size of the product is significantly larger in the presence of these compounds (Fig. 4a). The stabilization of CSF could occur, therefore, by additional phosphorylation of pp39^{mos} in the presence of ATP and NaF. Strictly speaking, however, CSF activity has been shown to be 'enhanced' by the addition of ATP and NaF during the first 24 h¹⁹. In practice, therefore, the additional phosphorylation may 'enhance' rather than merely stabilize CSF activity.

CSF activity can be sedimented from egg cytosol by centrifugation at 150,000g for 6 h²⁰. We also find that pp39^{mos} is selec-

tively sedimented under the same conditions (Fig. 4b). A wide spectrum of total protein is found in both pellet and supernatant fractions, but the pattern is predictably different between the two fractions (Fig. 4b). This also suggests that pp39^{mos}, which has a relative molecular mass of 39,000 (39K), is associated with other proteins, presumably as part of CSF. Collectively, these results both support the identity between pp39^{mos} and CSF, and also contribute to the understanding of the mechanism involved in calcium inactivation and ATP stabilization of CSF activity.

Discussion

According to Masui²⁰, who first demonstrated CSF in the amphibian unfertilized egg², any inhibitory factor responsible for the meiotic arrest of unfertilized eggs must satisfy the following four criteria: (1) the inhibitory factor must be absent in fertilized eggs; (2) it must be inactivated on activation of egg cytoplasm; (3) blastomeres injected with this factor must display the same arrest characteristics as found in unfertilized eggs; and (4) inhibition caused by this factor must be reversible. Amphibian CSF has all of these characteristics and is believed to be the endogenous meiotic inhibitor^{17,20}. We have shown here that the *Xenopus c-mos* proto-oncogene product also fulfills at least the first three criteria. We have demonstrated that: it is not present in fertilized eggs (Fig. 1); it is destroyed on egg activation both *in vivo* (Fig. 1, and accompanying paper³²) and *in vitro*³²; and microinjection of *c-mos* RNA into blastomeres of two-cell embryos specifically induces metaphase arrest and associated high MPF activity (Table 1 and Fig. 2). We have not directly addressed the fourth characteristic, but we have unequivocally demonstrated that pp39^{mos} is essential to CSF activity, by showing that CSF activity is abrogated by Mos-specific antibodies (Fig. 3 and Table 2). Moreover, we have shown that the properties of pp39^{mos} in CSF cytosol parallels and can explain the biochemical properties of CSF (Fig. 4 and accompanying paper³²). Taken together, we conclude that the *c-mos* proto-oncogene product, pp39^{mos}, is either the long-known endogenous meiotic inhibitor of amphibian eggs, CSF, or at

least a catalytic component of it. As an essential component, CSF activity, then, is dependent on the intrinsic kinase activity of the endogenous phosphorylated pp39^{mos} (accompanying paper³²) (Fig. 4b).

One apparent function of CSF is to stabilize MPF activity²¹⁻²³. Indeed, we have shown that MPF activity in *mos* RNA injected metaphase-arrested blastomeres is as high as in unfertilized eggs. At present, we do not know how the MPF activity is stabilized. If pp39^{mos} is the chief active component of CSF, however, one possible target in MPF is cyclin. Cyclin can apparently activate MPF by its association with p34^{cdc2} kinase¹⁰⁻¹², and its degradation at metaphase-anaphase transitions has been proposed to be responsible for the inactivation of MPF^{11,35}. Consistent with this proposal, Murray *et al.*²³ have recently demonstrated that a proteolysis-resistant mutant of cyclin not only prevents the inactivation of MPF but also arrests the cell cycle at metaphase in *Xenopus* eggs and embryos. We presume that pp39^{mos}, as CSF, may act to protect cyclin from its proteolytic degradation, thus stabilizing MPF activity and blocking the cell cycle at metaphase. Although pp39^{mos} may not be a component of MPF⁷, it could interact with cyclins. In this context, we note that polypeptides with similar molecular weights to those of cyclins are co-immunoprecipitated and heavily phosphorylated in the immune-complex Mos-kinase assay³².

Synthesis of pp39^{mos} is required for the activation of MPF and GVBD during progesterone-induced *Xenopus* oocyte maturation^{29,30}, whereas *mos* RNA injected into immature oocytes can induce the same two maturation events in the absence of progesterone³⁰. Based on these results, we proposed that pp39^{mos} may initiate meiotic maturation by 'activating' MPF³⁰. The actual function of the *c-mos* product, however, now appears to be the 'stabilization' of MPF activity, as discussed. The pp39^{mos} protein may, therefore, initiate meiotic maturation by altering the equilibrium²⁰ to favour 'stabilization' of active MPF. Consequently, the apparent 'activation' of MPF by pp39^{mos} may be explained by the stabilization of MPF. Except at the end of metaphase I when MPF is inactivated (see accompanying paper³²), we believe that the stabilizing function of pp39^{mos} works continuously throughout the maturation process and is active maximally in metaphase II-arrested unfertilized eggs (Fig. 5).

It has been postulated that CSF also plays an important role in embryonic mitosis^{22,35}, even though it has never been detected

after fertilization^{2,17,18} (Fig. 5). Similarly, pp39^{mos} is not detected soon after completion of meiosis and if it exists in early embryos, it does induce cleavage arrest. Although it is possible that transient pp39^{mos} expression occurs at undetectably low levels and may be required for each embryonic mitosis, it has been clearly demonstrated that cyclin is the only newly synthesized protein required for *Xenopus* embryonic mitosis³⁵. Thus, it seems that in early development, pp39^{mos} or CSF is not required for the embryonic cell cycle (Fig. 5).

In certain adult tissues, *c-mos* RNA transcripts have been detected at very low levels^{25,36,37}, but it is not known whether they are translated into a functional protein. The constitutive expression of normal Mos as well as viral Mos proteins in somatic cells induces cellular transformation³⁸. This expression is very low (0.0005% of total protein³⁹) and is similar to that in maturing oocytes (<0.001%, accompanying paper³²). It is possible that in somatic cells, Mos protein at such low levels may act to stabilize MPF activity, as in maturing oocytes, and lead to cellular transformation²⁹⁻³¹. When expressed at much higher levels (0.05% of total protein³⁹), however, Mos protein seems to arrest cell growth (our unpublished data) and eventually kills cells³⁹. This cytotoxic effect of viral Mos protein is reminiscent of the CSF function of pp39^{mos} in unfertilized eggs and *mos* RNA-injected blastomeres. We have been unable, however, to find evidence for metaphase arrest in these growth-arrested cells (our unpublished data). This may be due to the much longer G1 and G2 phases of somatic cells compared to maturing oocyte and cleaving embryos⁴⁰. In the longer phases, overexpression of Mos protein could stabilize MPF to a 'static' state and thus arrest progression of the cell cycle.

In almost all vertebrates, as in amphibians, unfertilized eggs remain arrested at metaphase II awaiting fertilization¹³ and in all vertebrates so far examined (human⁴¹, monkey³⁷, rat⁴², mouse⁴³, chicken³⁶ and frog²⁹), the *c-mos* proto-oncogene is expressed in germ cells or gonadal tissues²⁵⁻²⁸. In mice, *c-mos* maternal mRNA is degraded immediately after fertilization²⁶⁻²⁸ and, as in amphibians, Mos protein has not been detected in zygotes despite its presence in maturing and mature eggs³¹. Also as in amphibians, blastomeres of mouse two-cell embryos can be arrested at metaphase when fused with maturing oocytes⁴⁴. It is, therefore, almost certain that in mice and perhaps all vertebrates, the Mos-protein functions as an endogenous meiotic inhibitor or CSF. In invertebrates, however, unfertilized eggs are arrested predominantly at either the germinal vesicle stage (prophase I) or metaphase I, and in sea urchins, meiosis is completed before fertilization¹³. In these animals CSF or, for that matter, the *c-mos* gene has not been identified and it is possible that CSF or the *c-mos* gene may be specific only for vertebrates. Alternatively, the *c-mos* gene may exist at least in some invertebrates but its expression (or action as CSF) may be regulated differently to induce the premature meiotic arrest at prophase I or metaphase I. As with cyclins^{11,45,46}, Mos proteins show high sequence divergence among known animals species^{29,37}. This could be explained if cyclin or some other evolutionarily divergent protein(s) were substrate(s) of the Mos-kinase activity. □

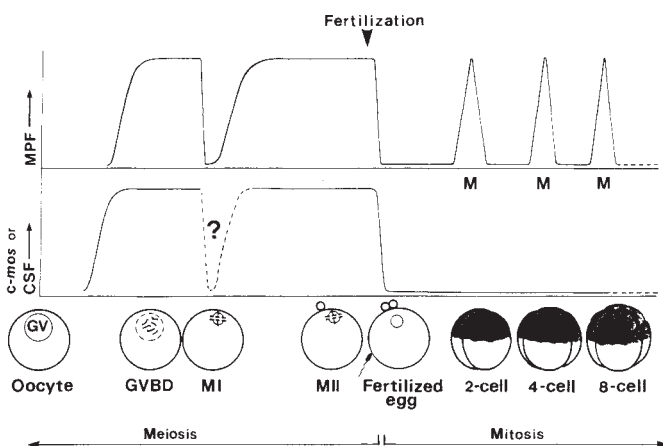


FIG. 5 MPF and CSF (c-Mos) levels during early *Xenopus* development. MPF levels rise and fall during meiosis as well as during early embryonic mitoses, whereas CSF (c-Mos) levels are detectable only during meiosis with possible fall just after meiosis I. Thus, CSF is postulated to stabilize MPF activity in a meiosis-specific manner. See the text for further details. GV, germinal vesicle; GVBD, germinal vesicle breakdown; M, mitosis; MI, mitosis I; MII, mitosis II.

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LETTERS TO NATURE

GS2023+338: a new class of X-ray transient source?

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TRANSIENT X-ray sources are generally considered to comprise accreting binary systems containing either a black hole or a neutron star^{1–5}. The transient X-ray emission is thought to result from episodic mass transfer to the compact star; if this is so, transient sources should provide information on the mass-accretion mechanism. On 21 May 1989, the All-Sky Monitor⁶ (ASM) onboard the satellite Ginga⁷ discovered an unusual bright X-ray transient source, GS2023+338, in the constellation of Cygnus⁸. The ASM and Large-Area Counters⁹ (LAC), also on Ginga, have since monitored the source. Here we report the light curves and spectral evolution observed by the ASM and LAC. From 21 May to 1 June, the intensity varied by up to a factor of 500 on timescales from a few seconds to a few days¹⁰. Subsequently the rapid variations persisted, whereas slower ones disappeared. The spectral data fit a power-law model with a photon index of 0.0 to –2.5 and an absorption column density of $10^{21.7}$ – $10^{22.8}$ H atoms cm^{–2}. This unusual behaviour remains to be explained.

Some X-ray transient sources have ‘ultra-soft’ (low-energy) spectra¹¹. The mass of one such source, the X-ray star in AO620–00, has been estimated to be greater than $3 M_{\odot}$ (ref. 12), indicating that ultra-soft sources may contain black holes¹³. Other X-ray transient sources may be classified into two categories^{14,15}, soft and hard; these are thought to contain a neutron star. Some soft transient sources exhibit X-ray bursts^{16–18} and have been identified optically as K or M dwarfs¹⁹; these are therefore considered to be low-mass X-ray binaries. Hard transient sources are generally X-ray pulsars, and are thought to be massive X-ray binaries with eccentric orbits²⁰.

The ASM consists of a pair of proportional counters filled

with a Xe–CO₂ gas mixture. Each proportional counter is divided into three independently operating chambers with an effective area of 70 cm². Each chamber has a fan beam collimator (1°×45°) slanted at different angles with respect to the z-axis of the satellite. The entrance window is beryllium, 50 μm thick. For normal scanning observation of the ASM, the satellite performs a 20-min rotation, once a day. The ASM usually observes X-rays in the range 1–20 keV with 16 energy channels and with 0.5-s time resolution. The position of the X-ray source can be determined to within 0.5°. The LAC comprises eight identical proportional counters with 62-μm thick beryllium, filled with an Ar–Xe gas mixture. The total effective area is 4,000 cm². The maximum time resolution is 1 ms.

On 21 May 1989 we discovered, during routine monitoring, a transient X-ray source in the constellation of Cygnus. Unfortunately we did not observe the Cygnus region between 11 May and 21 May because of the Earth's occultation of the Cygnus region during satellite rotations. On 11 May the upper limit of

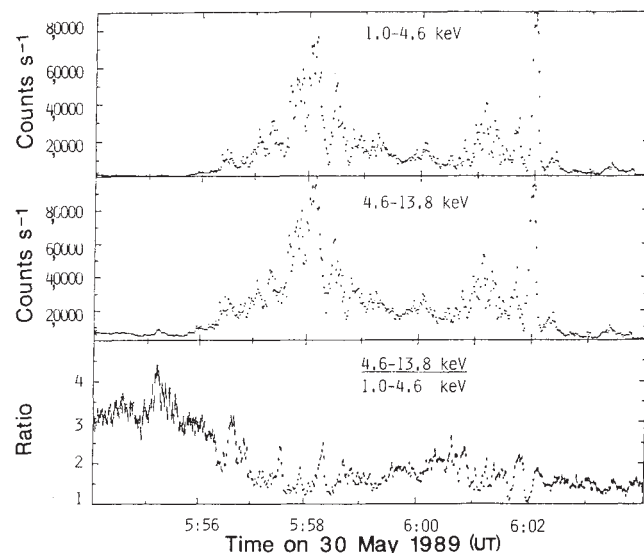


FIG. 1 The background-subtracted and dead-time-corrected light curves observed with the LAC on 30 May with a bin width of 1 s. The energy range is 1.0–4.6 keV and 4.6–13.8 keV. The hardness ratios are also plotted. At the peak count rate, the dead-time correction factor was ~6.

the flux in the energy range 1–6 keV was ~ 50 mCrab (1.0×10^{-9} erg s $^{-1}$ cm $^{-2}$). We determined⁸ the source position using data from a LAC Scan (23 May) and from the ASM data obtained on 21 May and designated the source as GS2023+338. We used triangle fitting of the light curves, observed through five different collimators (the data obtained through one collimator was contaminated by X-rays from Cygnus X-3), as a function of satellite azimuth.

After our report⁸ V404 Cyg was identified to be an optical candidate for GS2023+338 (refs 21, 22). Its position (right ascension (1950) = $20^{\text{h}} 22^{\text{m}} 6.30^{\text{s}} \pm 0.03^{\text{s}}$ and declination (1950) = $33^{\circ} 42' 16.7 \pm 0.4''$) is just outside the area we reported⁸. Because both the ASM and the LAC have slat collimators of $\sim 1^{\circ}$ (full width at half maximum) on the narrow side and the observation time for each detector of the ASM is ~ 3.3 s in the normal scanning mode, the position accuracy is strongly affected by source intensity variations on such a timescale. When the LAC observed this source on 28 May 1989, the intensity varied by factor of 500 on a timescale of at least a few minutes¹⁰. This made it difficult to determine the position with X-ray data. Using the ASM data obtained on 23 May, which was one order of magnitude brighter than that obtained on 21 May, and the data obtained from several scans of the LAC on 23 May, the determined area includes V404 Cyg.

Figure 1 shows light curves in the energy range 1.0–4.6 keV and 4.6–13.8 keV observed with the LAC on 30 May. The hardness ratio varies widely on timescales from seconds to minutes. The intensity also varied by a factor of 500 (up to 17 Crab). Data with a 1-ms time resolution were also obtained on 30 May. Power spectra in the frequency range 0.0156–500 Hz do not show clear coherent pulsation but significant variations up to the 500-Hz range were found. The detailed results from the LAC observations will be published elsewhere.

The long-term light curves observed with the ASM in the energy range 1–6 keV and 6–20 keV and their ratios are shown in Fig. 2. On 21 May, the intensities in the energy range 1–6 keV and 6–20 keV were ~ 0.1 Crab and 1 Crab, respectively. This means that the data showed an extremely 'hard' spectrum. On 22 May, the intensity became 4 Crab and 3.5 Crab in the energy range 1–6 keV and 6–20 keV, respectively indicating that the spectrum was becoming softer. After 23 May, the intensity decreased rapidly and on 26 May it was less than 80 mCrab in the energy range 1–6 keV. (This value is larger than the nominal detection limit of the ASM⁶, because the source was near edge of the collimator.) After 1 June, significant fluxes were again detected and the spectrum became hard. The flux was ~ 100 –200 mCrab in the energy range 1–6 keV and the violent variation

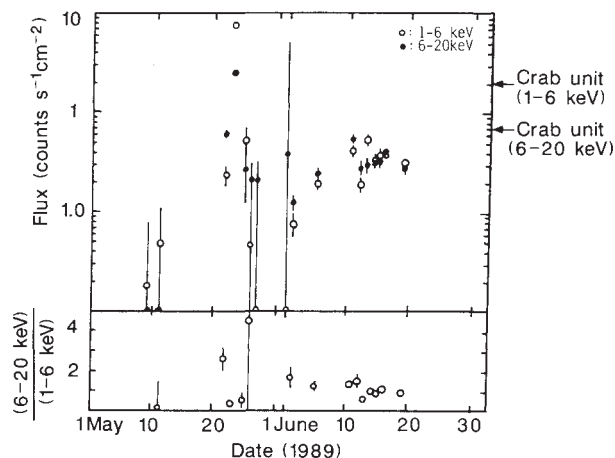


FIG. 2 The long-term background-subtracted light curves of the energy range 1–6 keV and 6–20 keV observed with the ASM. The hardness ratios are also plotted. Each datum is the averaged value of the intensities observed by the six chambers and each chamber observes for ~ 3.3 s per scan.

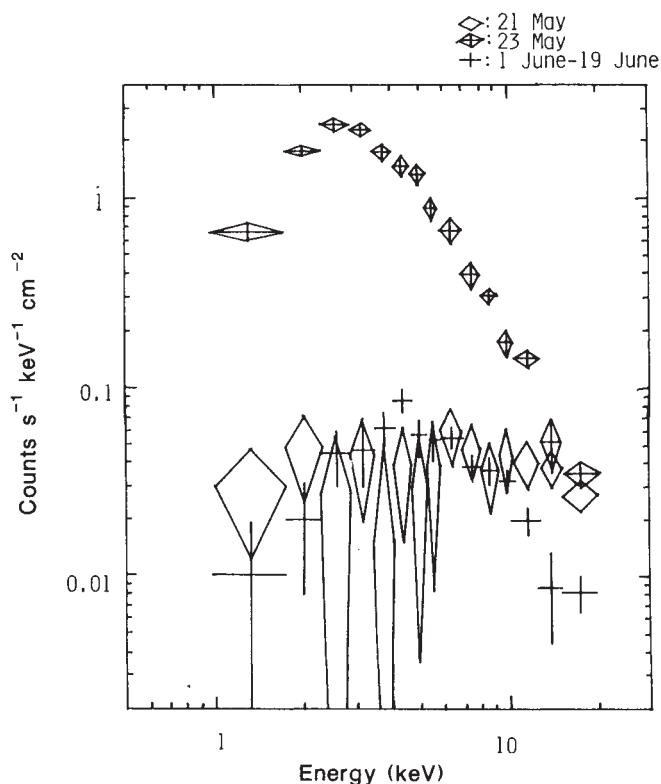


FIG. 3 The spectra observed with the ASM on 21 May, 23 May and the averaged data obtained between 1 June and 19 June.

on the timescale of a day was not seen. Rapid variations on a timescale of less than minutes still remained, however, which were observed with the LAC. The hardness ratio shows gradual softening. The data shown in Fig. 2 were observed by the ASM with normal scanning. Each detector of the ASM observed for only 3.3 s, and the observed time-span with six detectors ranges from several seconds to several tens of seconds. Therefore, the data shown in Fig. 2 represent just 'snap-shots' of this violently variable source.

The spectra obtained with the ASM on 21 May and on 23 May, and the averaged data obtained between 1 June and 19 June are shown in Fig. 3. A power-law model including interstellar absorption²³ can be fitted to all spectra within the error. Between 24 May and 1 June, we could not get significant spectral data. The energy spectrum obtained on 21 May was very unusual. When we simulate this spectrum with a power-law model, the photon index is $+0.000 \pm 0.50$ with an interstellar absorption of $10^{21.7 \pm 1.6}$ H atoms cm $^{-2}$. On 23 May the spectrum became softer and was represented by a power-law model with a photon index of -2.5 ± 0.16 and an interstellar absorption of $10^{22.36 \pm 0.13}$ H atoms cm $^{-2}$. After 1 June, the spectra became harder again; one spectrum had a photon index of -1.4 ± 0.4 and an absorption column density of $10^{22.8 \pm 0.3}$ H atoms cm $^{-2}$. (The detailed spectra observed with the LAC showed more complex feature and extreme variability.)

GS2023+338 cannot be classified into any of the three known groups of the transient X-ray sources. The spectrum of an ultra-soft transient source is far softer than the spectrum of this source. The spectrum of soft transients is similar to that of low-mass X-ray binaries, which is represented approximately by the combination of a 1-keV disk black-body spectrum²⁴ and a 2-keV black-body spectrum. The transient source reported here has a harder spectrum than do soft transient sources. The spectra of hard transient sources are represented by a power law with an exponential cut-off at ~ 10 keV. At present, it is hard to say anything about the high-energy cut-off in the spectra. The

hard transient sources are generally X-ray pulsars, however, and we have not yet found any regular pulsation. The time variation up to the millisecond range in the light curve seems to be random, which resembles a black hole candidate Cyg X-1 (refs 25, 26). This complex behaviour of the spectrum and time variation is unusual in an X-ray transient source. □

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The rotating envelope of the hot star gamma Cassiopeiae resolved by optical interferometry

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CERTAIN hot stars, belonging to the Be class, may have an envelope of hydrogen gas, possibly in the form of a rotating disk¹ or spheroidal shell². Other models involve elliptical rings³ or close binary systems where the Roche lobe of the companion is filled with hydrogen². The angular size of these features is too small for direct detection by conventional telescopes, and attempts to resolve the structure using speckle interferometry (a technique that restores the diffraction-limited resolution otherwise degraded or spoiled by the atmosphere) have failed. The various models of the structure of the hydrogen envelope are based on spectroscopic data, together with polarization and variability measurements. After a century of spectroscopic observations, which showed considerable but little-understood variations, the hydrogen envelope of the star gamma Cassiopeiae was angularly resolved by the prototype interferometer I2T in 1986 (ref. 4). Here we report observations from its successor GI2T, which show further high-resolution details of the hydrogen envelope. The data clearly show the envelope in rotation and approximately fit a disk model. Thus, the GI2T yields optical information capable of constraining astrophysical models on a milliarcsecond scale.

The GI2T^{5–7} consists of two 1.5-m telescopes that are moveable along a railway track. The track is oriented north–south, horizontal at latitude 43° 45' N, 70-m long, and is expandable to 300 m. The telescopes steer light towards a common Coudé focus, where the images are recombined. The interference pattern, similar to Young's fringes, is analysed through a grating spectrograph^{5,6}, with a spectral resolution of 1.5 Å. The spectrum

is recorded digitally in the photon-counting mode with a CP40⁸ intensified-CCD (charge-coupled device) camera providing 570 × 770 pixels. This system is a forerunner of telescope array systems^{9,10} using the aperture-synthesis techniques of radio-astronomy to improve the imaging resolution beyond the milliarcsecond range. Although three or more telescopes are normally required for aperture synthesis through the fluctuating Earth atmosphere, two can suffice if the object includes a point source usable as a phase reference. Pending the completion of a third telescope, we have used the stellar photosphere within the envelope of γ-Cas as a reference source to obtain spatial information on the envelope structure. Although the present data do not yet provide enough baseline coverage, synthesis maps seem achievable in such cases.

On unresolved sources, the fringes appear as nearly parallel bright lines across the spectrum, with a slope proportional to the optical path difference¹¹. The slope was kept constant by stabilizing the optical path difference with 4-μm accuracy. For resolved sources, the fringe-visibility modulus and phase measured for a given baseline and wavelength provide one complex Fourier component of the object's intensity distribution. Any shift of fringe position (phase) at a given wavelength in the spectrum indicates a position difference in the object, along the direction of the baseline. The corresponding angular sensitivity is expected to reach 3 μas with 300-m baselines, because phase shifts of 3 or 4° seem to be detectable^{12,13}.

On objects such as γ-Cas, the unresolved photosphere observed in the continuum provides a phase reference. We expect this to allow the reconstruction of high-resolution images. In the case of γ-Cas, however, the star envelope rotates and varies on timescales of hours, which is shorter than the several nights required to complete aperture-synthesis observations that require many baselines. Therefore we can only obtain time-averaged images, until a many-telescope system such as the Optical Very Large Array⁹ becomes available. Because few baselines are involved in our present data on γ-Cas we cannot present a synthesis image. Clear evidence for a rotating envelope is, however, present in the visibility spectra (Fig. 1), although its shape is unknown.

We recorded ~300,000 camera images, each exposed for 0.02 s to freeze the effect of the atmospheric turbulence, of γ-Cas during the nights of 27–30 December 1988. We digitally processed each image, which contained ~100 photon events, using correlation techniques. The results were co-added to reduce the effect of atmospheric 'seeing' and photon noise, according to the principle of speckle interferometry^{14,15}. This principle, which applies to single- or multiple-aperture systems, preserves the high-resolution information in the presence of atmospheric turbulence which otherwise limits the resolution of telescopes with apertures larger than 10 cm.

The high visibility modulus and the uniform phase observed in the continuum confirmed that the source of the continuum emission is mainly the barely resolved photosphere of the star, in agreement with the 0.44-mas size assumed in most models. We used this source as a phase reference.

Algorithms (D. Mourard, thesis (Univ. Nice) in preparation) developed from those of Thom^{4,16} gave the visibility modulus and phase in each spectral channel, relative to a reference channel located in the continuum (Fig. 1). We isolated channels of optimum width, 3.6 Å for the modulus and 1.5 Å for the phase, by applying windows to the recorded exposures. In a given channel, the modulus was calculated by autocorrelating each exposure, accumulating the results over successive exposures, Fourier transforming them and dividing the amplitude of the peak appearing at the fringe frequency by the amplitude similarly obtained from a reference window in the continuum (6,527 Å), the width of which was adjusted to give equal total photon count.

For calculating the phases we did two-dimensional cross-correlations of two channels in each exposure: the desired

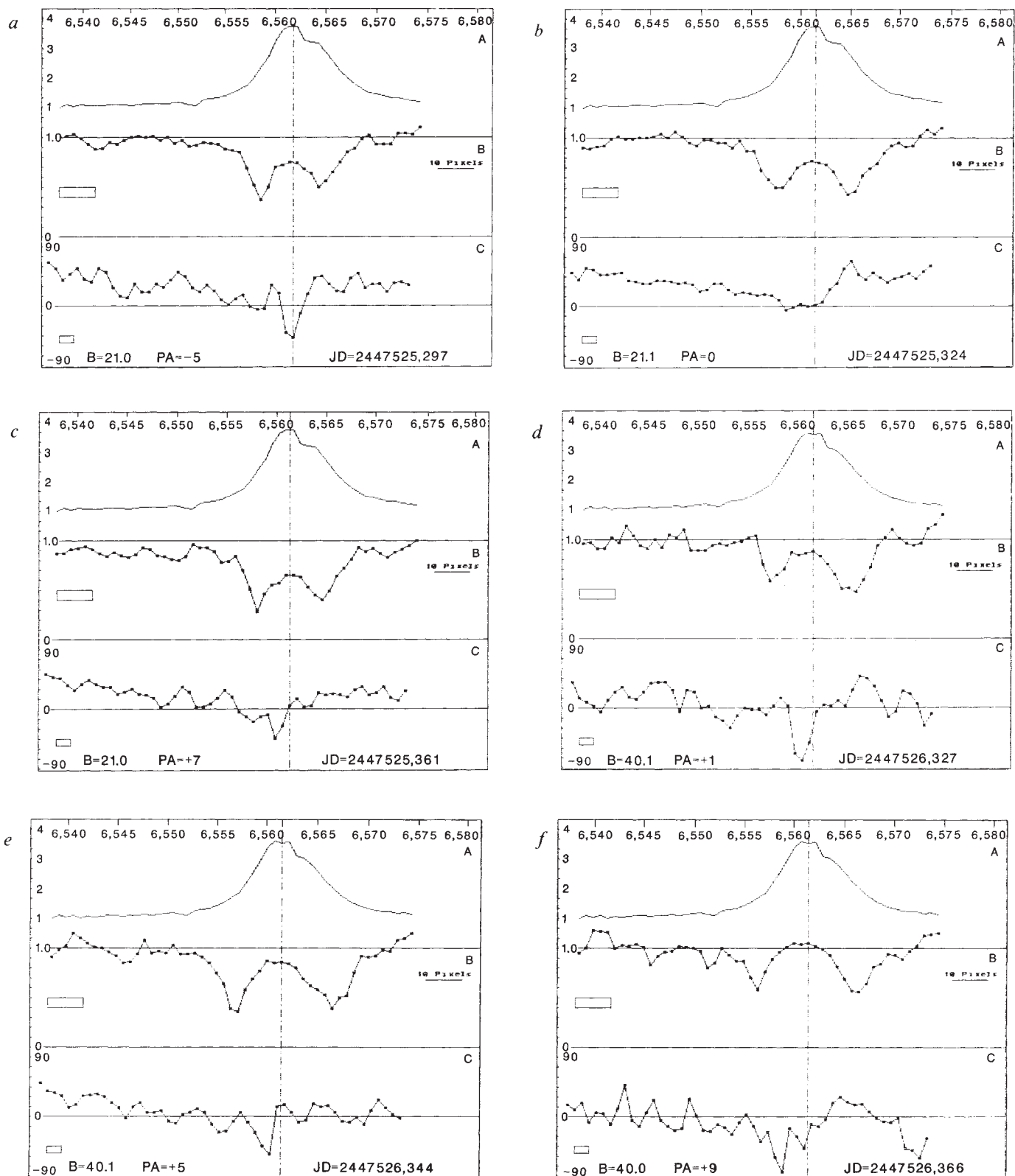


FIG. 1. Spectrum (A), with relative visibility modulus (B) and phase (C) as a function of wavelength, across the hydrogen alpha emission of γ -Cas. *B* (in metres) is the baseline projected on the sky, *PA* (in degrees, from north to west) the position angle of the baseline and *JD* the Julian date of the observation. The visibility drop in both wings of the line is evidence for a hydrogen structure rotating with high velocities. Among the noisy phase curves, a significant disturbance also occurs at the line position. Because

the line profile varied markedly from night to night, differences between corresponding curves may be linked to the changing morphology. The uncertainty boxes shown indicate, vertically, the r.m.s. noise level and, horizontally, the width of the sliding window used to reduce the spectral resolution. The signal-to-noise ratio is approximately twice as good at the centre of the line, owing to the higher photon count.

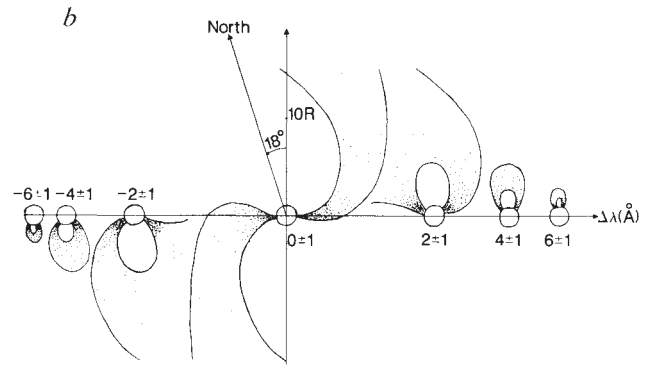
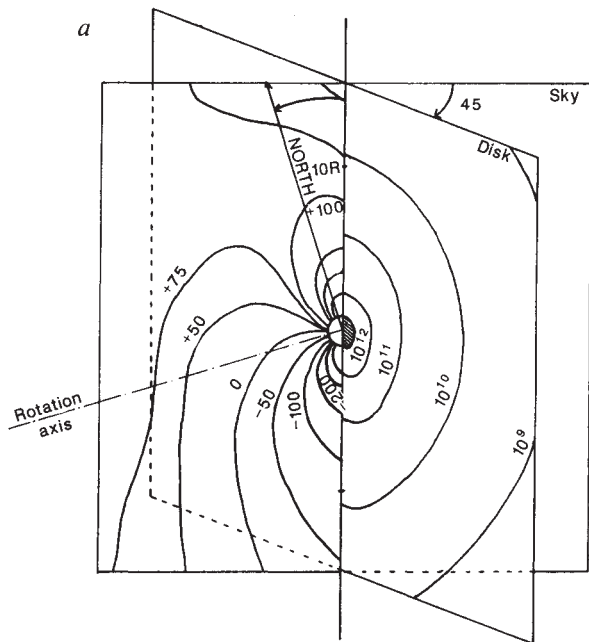


FIG. 2 The rotating hydrogen disk of γ -Cas, according to the model of Poeckert *et al.*¹. *a*, Geometry of the thin disk: R is the star radius. In the disk plane, contours of hydrogen density are indicated in cm^{-3} . In the sky plane, contours of radial velocity are given in km s^{-1} . *b*, Expected object shapes in contiguous spectral channels, $2 \text{ \AA}$ wide, across the $\text{H}\alpha$ line. The binary shapes cause dips, like those observed, in the modulus spectra. Interpreted in this way, the observations are somewhat consistent with the disk model. Refined fitting requires a better knowledge of luminosity distributions.

spectral channel and a reference channel located in the continuum between 6,516 and 6,538 $\text{\AA}$. We accumulated the result over successive exposures and then Fourier transformed it. The complex phase of the peak obtained gave the difference between phases in the selected spectral channel and the reference channel.

The modulus spectra show a pair of dips in the wings of the $\text{H}\alpha$ line. We have compared their position, at the different baselines, with values derived from the model of Poeckert and Marlborough¹. This model involves a hot star at a distance of 200 pc, surrounded by a rotating disk of hydrogen which is inclined at 45° on the line of sight. The disk should be seen as an ellipse. Polarization data¹⁷ indicate that the position angle of its major axis is 18° (Fig. 2a). With these assumptions, the object's appearance at each wavelength within the hydrogen line includes the star and one of the crescent-shaped contours of radial velocity (Fig. 2b). If projected on the baseline direction, this pattern resembles a double source, which can account for the dips observed in the visibility spectra. We interpret them as implying a half-fringe spacing for both sources. The spacing amounts to 3.2 mas at 21 m and 1.7 mas at 40-m baseline, which translates respectively to 14.5 and 7.7 stellar radii at the 200-pc distance.

We compare these values with the elongations, in the sky

plane, of the corresponding radial velocity zones given by the model (3.2–12.8 and 2.5–7.5 stellar radii respectively). The agreement with the model seems reasonably good, considering that the location of the photocentre in the crescent-shaped contour is highly dependent on the luminosity distribution along the contour. In principle, this uncertainty can be reduced by deriving the position of the crescent's photocentre from the monochromatic luminosity distributions given by the model. If such distributions become available, they may also be Fourier transformed to evaluate the agreement between the observed phase spectra and the model. A characteristic peak and dip which indicate a rotating envelope (ref. 12, D. Mourard, thesis (Univ. Nice) in preparation) can be seen on the phase curves, but its amplitude is lower than expected from preliminary estimates. From the phase spectra the receding part of the disk is south of the star, in agreement with the I2T observation⁴.

A new 1.5-m telescope soon to be tested as a prototype for the future Optical Very Large Array⁹ is expected to provide a third element for the existing array. With three and more elements, high-resolution images of γ -Cas and other poorly understood objects should become systematically available. Stellar interferometry from the ground can reach stellar magnitude 10 with 1- $\text{\AA}$ resolution and 15 with 100 $\text{\AA}$ (ref. 15). Work in the infrared and near ultraviolet is also being considered. $\square$

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Precise single-grain $^{40}\text{Ar}/^{39}\text{Ar}$ dating of a cold to warm climate transition in Central Europe

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RECENT advances in $^{40}\text{Ar}/^{39}\text{Ar}$ dating^{1,2} have made it possible to date individual K-feldspar grains from Pleistocene tephra, a capability that greatly improves the reliability and temporal resolving power of the method. Here we apply these new techniques to the dating of a phonolite tephra from the East Eifel volcanic field in West Germany, which is sandwiched between loess and palaeosol (alfisol) deposits, and which was therefore erupted during the transition from a glacial to an interglacial period. Our age estimate for this transition is 215 ± 4 kyr (1σ), which has important implications for the marine $\delta^{18}\text{O}$ timescale and for models of global climate change during the Pleistocene. The results show that single-grain dating can detect and compensate for the large quantities of xenocrystic contaminants which are found in many tephra deposits. This technique could be used to date the tephra layers found in marine sediment cores and the results could greatly enhance the reliability of the marine $\delta^{18}\text{O}$ timescale for more rigorous Fourier analysis testing of the Milankovitch hypothesis.

We have reported² laser single-grain $^{40}\text{Ar}/^{39}\text{Ar}$ dates for several near-vent tephra deposits from the East Eifel Volcanic Field (EEVF), including a tephra from the rim of Wehr volcano (Fig. 1, site 1, Dachsbusch sand pit). We found this Hüttenberg tephra (H tephra) to be 213 ± 4 kyr old. We have since found seven other isolated occurrences of the H tephra over an area of some 400 km² (sites 4–10). We have used geochemistry, phenocryst and xenocryst assemblages, stratigraphy and, for seven of the eight sites, $^{40}\text{Ar}/^{39}\text{Ar}$ geochronology, to demonstrate that these deposits all represent the same eruption of Wehr volcano that is expressed at site 1.

Details of the identification of the H tephra by physical and chemical means will be given elsewhere. Briefly, the H tephra

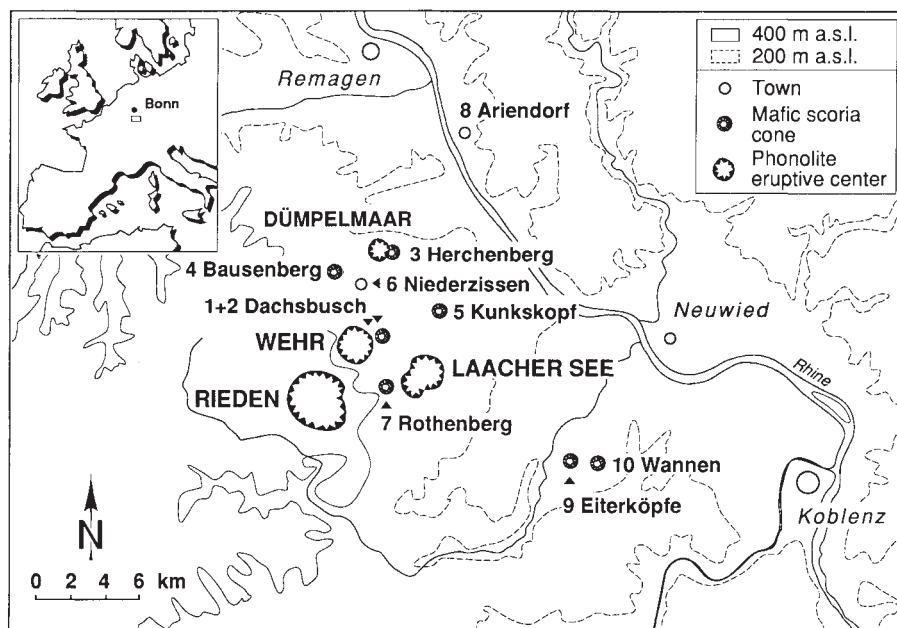
is a collection of Plinian fallout pumice beds and ash-flow deposits (sites 4, 5, 6, 10), as well as phreatomagmatic base surge (sites 1, 7) and fallout deposits (sites 8, 9) the xenolith suite of which (abundant mica schists and lack of basanite xenoliths) is unique in the EEVF. The H tephra's phenocryst assemblage includes K feldspar (anorthoclase), plagioclase, clinopyroxene, amphibole and sphene. Feldspathoids, however, are totally absent, a feature we have not seen in any other phonolite tephra in the EEVF, thus making the phenocryst assemblage another highly diagnostic identifying criterion.

The chemical compositions of all H tephra essential lapilli define a single differentiation path with, for example, systematically increasing concentrations of incompatible trace elements (such as zirconium, niobium, thorium and hafnium) towards highly evolved members of the eruption. Also, chemically zoned composite tephra sections (sites 5 and 6) demonstrate that the H tephra represents a strongly compositionally zoned eruption that started with highly evolved, silica-undersaturated phonolite, but ended with mafic, silica-saturated trachyte magmas.

The stratigraphic sections of the sampled sites are shown in Fig. 2. The more distal sites, especially Ariendorf (site 8), show the complete loess-tephra-palaeosol sequence particularly well. There is typically a humus-rich granular soil layer ('A horizon') lying immediately above the H tephra and a pedogenetic clay-rich loam ('B horizon') is developed in the loess directly below. There is no loess present just above the tephra and no humus-rich soil immediately below. It is important to note that the formation of the 'B horizon' below the tephra resulted from leaching of the overlying 'A horizon' during the interglacial and thus it post-dates both the tephra and the underlying loess. This point is made clear at sites 4, 6 and 7 where the 'B horizon' of the soil profile is absent because the tephra was too thick for the leaching solutions to penetrate.

At sites 1, 4, 5, 7, 9 and 10 the H tephra and palaeosol were protected by overlying scoria cones, but this was not the case at site 6 where erosion had removed the palaeosol layer. The older palaeosol at site 7 (Rothenberg) formed on 400–450 kyr leucite-phonolite tephra (unpublished results), indicating a major erosion break in the Rothenberg section. At site 1, the base of the H tephra is not exposed, and therefore the presumed tephra-loess contact cannot be seen. Despite these facts, all of the H-tephra sites are stratigraphically consistent and strongly suggest that the H tephra was deposited at the end of a glacial period, during the brief interval of transition to a warmer climate.

FIG. 1 Location map for the sites studied. Only a small fraction of the mafic scoria cones of the East Eifel volcanic field are shown.



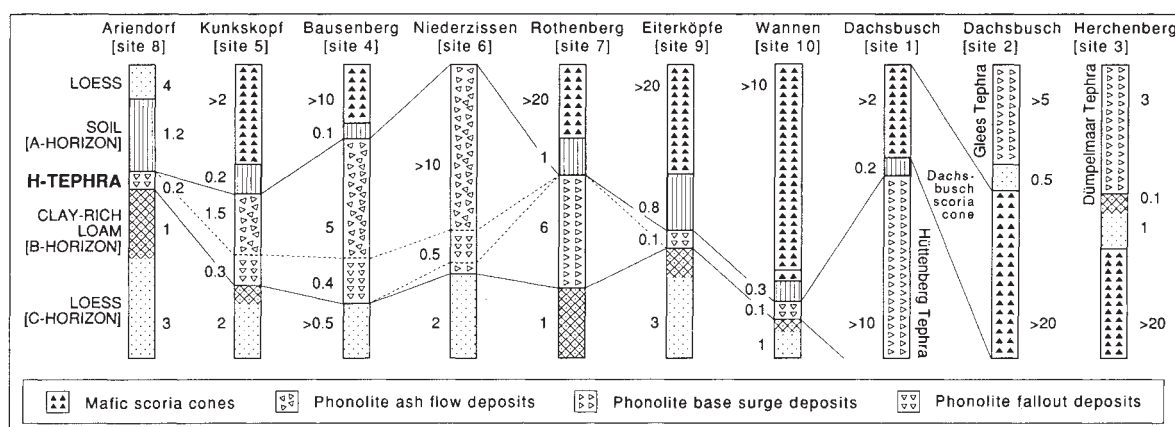


FIG. 2 Stratigraphic sections studied. Solid lines correlate main boundaries and dashed lines correlate subunits within the H tephra. Numbers are

thicknesses in metres. The names of sites 1, 4, 5, 7, 9 and 10 refer to the mafic scoria cones overlying the H tephra.

The results of the $^{40}\text{Ar}/^{39}\text{Ar}$ analyses from 105 laser fusions of K feldspars from H-tephra essential lapilli are shown in Fig. 3a, b in the form of Ar isotope correlation diagrams. In a few cases where grain size was small, multiple grains (≤ 10) were fused, but the vast majority of the analyses were carried out on single ~ 1 -mm-sized crystals. A small number of analyses with high Ca/K ratios (that is, $\text{Ca}/\text{K} \geq 0.5$, derived from $^{37}\text{Ar}/^{39}\text{Ar}$) were omitted, as these were likely to have been contaminated by plagioclase or some other calcium-rich phase.

Figure 3a includes the results from sites 1, 5, 6 and 9 which are the most mafic samples. The isotope ratio points are nearly collinear, and 27 of 33 analyses define an isochron with an initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of 293.4 ± 2.3 and an age of 215 ± 4 kyr (mean squares of the weighted deviations (MSWD) = 1.58, all uncertainties 1σ). We consider this value to be our best estimate for the eruption age of the H tephra.

The mafic results contrast dramatically with the 72 analyses from the most differentiated samples (sites 4, 7, 8, Fig. 3b). The data are, within measurement uncertainties, bounded by the isochron line derived from Fig. 3a, indicating that these samples

are indeed contemporaneous with the mafic units. However, a large fraction of the points have apparent ages that are much higher than the eruption age. These points do not show a trend towards a single high value of $^{40}\text{Ar}/^{36}\text{Ar}$ which would be expected if the erroneous ages were caused by a single anomalously high value of $(^{40}\text{Ar}/^{36}\text{Ar})_{\text{initial}}$. Thus, if these ages are due to excess ^{40}Ar , then each grain that does not lie on the eruption isochron must have been contaminated by its own unique composition of initial argon. It seems more likely that the discordant feldspars are indeed older xenocrystic minerals, derived from subsurface equivalents of Tertiary and Pleistocene Eifel volcanics, which were incorporated into the magma before eruption, retaining some or all of their original ^{40}Ar .

The large difference in the fraction of xenocrysts in the two groups of H-tephra samples (as manifest in Fig. 3a, b) is probably due to the different quantities of genuine phenocrysts in the mafic and differentiated units. Phenocrysts are abundant in the mafic lapilli whereas the differentiated lapilli are nearly aphyric. Thus, a small absolute concentration of contaminating xenocrysts in the magma could dominate the total suite of K feldspars

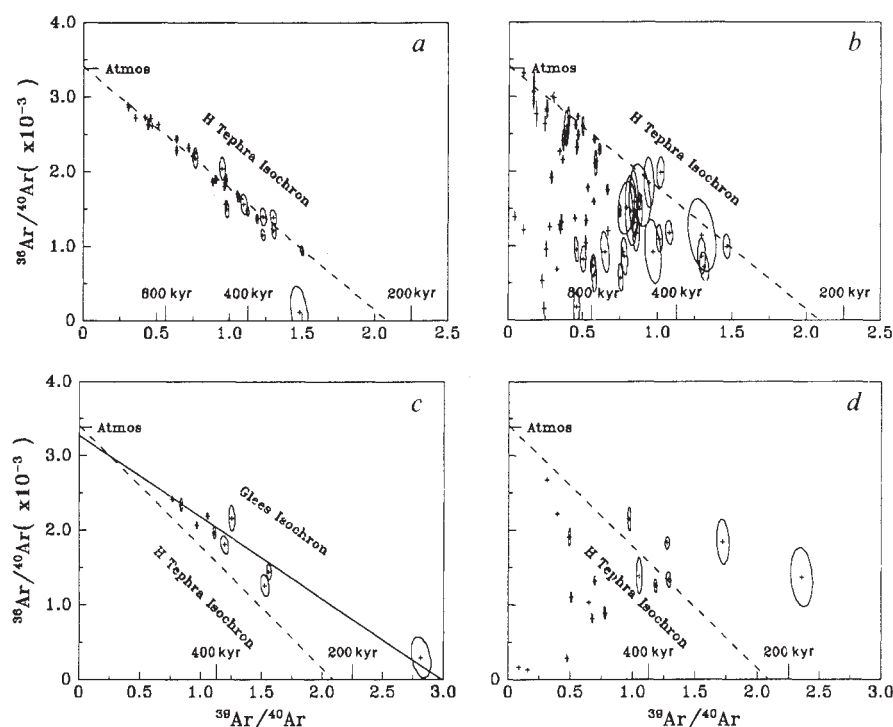
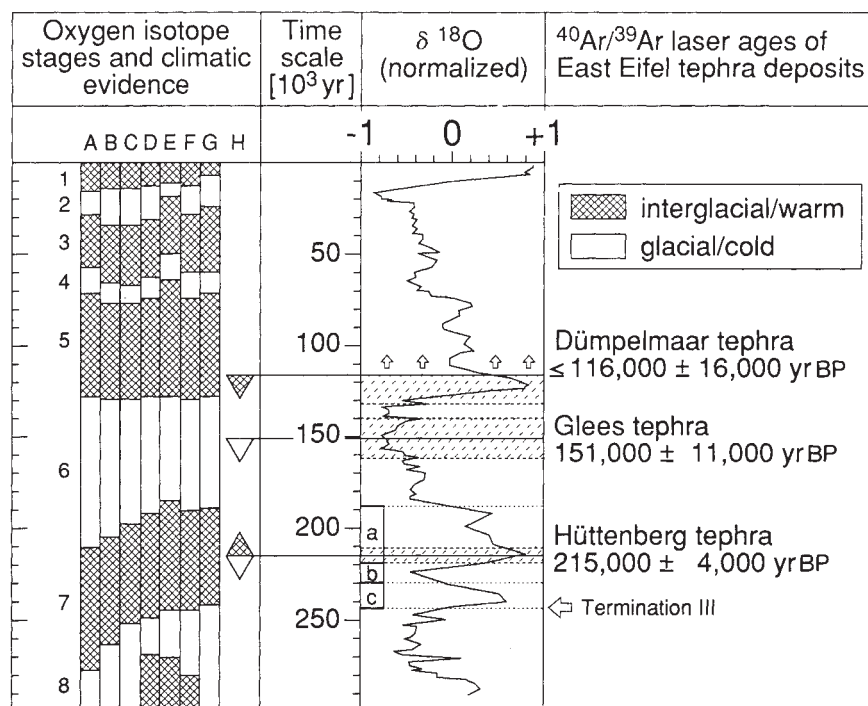


FIG. 3 Ar isotope correlation diagrams ($^{36}\text{Ar}/^{40}\text{Ar}$ plotted against $^{39}\text{Ar}/^{40}\text{Ar}$) with measured values marked by crosses and surrounded by their estimated 1σ uncertainty ellipses. Atmos, is the composition of atmospheric Ar and the ages marked correspond to x-axis intercepts at various age values. All values were normalized to the same irradiation conditions. Dashed lines labelled 'H-Tephra Isochron' are the isochron from a. a, Mafic H-tephra units, sites 1, 5, 6, 9. b, Differentiated H tephra at sites 4, 7, 8. Older xenocryst values lie below and to the left of the H-tephra isochron (younger). c, Glees tephra results. All points are above and to the right of the H-tephra isochron. Solid line, Glees isochron with $n=8$, age = 151 ± 11 kyr, $^{40}\text{Ar}/^{36}\text{Ar}_{\text{initial}} = 305 \pm 11$, MSWD = 1.74. d, Dümpeimaar tephra results: three samples are significantly younger than H-tephra isochron.

FIG. 4 Terrestrial palaeoclimatic evidence and laser $^{40}\text{Ar}/^{39}\text{Ar}$ ages plotted against several marine palaeoclimate timescales. Dark boxes and triangles mark warm (soil-forming) climatic conditions; white boxes and triangles mark glacial periods. Stage 7 substages marked a, b, c. Oxygen isotope record from ref. 6. Column legend: A, ref. 8 (CARPOR); B, ref. 9; C, ref. 10; D, ref. 11; E, ref. 8 (STUNE); F, ref. 12; G, ref. 13; H, this study.



in the differentiated units but be hardly noticeable in the phenocryst-rich mafic samples.

Previous workers have correlated the H tephra at Ariendorf (site 8) with other plagioclase phonolite eruptions in the EEVF (Glees tephra of Wehr volcano, Dümpelmaar tephra)^{3,4}. We have studied the near-vent Dümpelmaar (site 3) and Glees tephra (site 2) and have found that both are distinct from the H tephra in geochemistry, xenolith and phenocryst suite. For example, they lack mica schists and contain feldspathoid phenocrysts, both features that are incompatible with the tephra at Ariendorf. Furthermore, none of the 24 Ariendorf $^{40}\text{Ar}/^{39}\text{Ar}$ ages is incompatible with an eruption age of 215 kyr, but, as shown in Fig. 3c, d, all of the Glees samples and 3 of the Dümpelmaar samples give ages significantly younger than 215 kyr.

Eight of the ten argon analyses of Glees tephra define an isochron with an age of 151 ± 11 kyr and its stratigraphy indicates that it erupted during, or at the end of, a glacial period. The Dümpelmaar tephra is heavily contaminated by xenocrysts and we do not yet have sufficient data to define its eruption age precisely. So far, the two lowest-age analyses only indicate that this tephra was erupted $\leq 116 \pm 16$ kyr BP (before present). The stratigraphic section at site 3 indicates that the eruption occurred during, or at the beginning of, an interglacial warm period. Because the Hüttenberg and Glees tephra of Wehr volcano, plus the Dümpelmaar and the 11,000 yr BP Laacher See tephra are the only plagioclase-bearing phonolite eruptions known from the EEVF, we feel that the tephra at Ariendorf is in fact a distal version of the H tephra.

Figure 4 shows our age data in the context of several different palaeoclimatic timescales from the late Pleistocene. There seem to be only two possible locations for the H tephra within these schemes: either at the beginning of warm substage 7a within interglacial stage 7 (refs 5, 6), or at the very beginning of interglacial stage 7 (termination III), with the loess underlying the H tephra representing stages 8 plus 7b, or stage 8 only. If the first correlation is correct, then our ages for the H Glees and Dümpelmaar tephra are in substantial agreement with several of the recent palaeoclimatic timescales (such as Fig. 4 columns D, E, F, G). Soils formed during substage 7c are

apparently absent in the Eifel, however, with the possible exceptions of sites 1 and 7. This means that either stage 7c was not developed in the climate of Central Europe, or that soils formed during 7c were not preserved in at least six of the sections studied. The second correlation implies that either all of the marine-based timescales in this age range are too old by at least 30 kyr, or that oxygen isotope stages 7b and 7c do not reflect global interglacial climatic conditions.

In either case, our results are distinctly younger than recent U-Th disequilibrium age estimates by Winograd *et al.*⁷ whose date for the stage 7–8 boundary was 272 kyr and whose 7b–7a boundary age was ~ 240 kyr (7b = 248 kyr).

Our study exemplifies the potential of tephrochronology in linking marine and terrestrial palaeoclimate records. It also vividly demonstrates the advantages of single-grain dating over traditional bulk methods. The total integrated age derived from combining all mafic H-tephra samples is 217 kyr which is not significantly different from our eruption age estimate. The same calculation for the differentiated H-tephra samples gives a bulk age of 445 kyr which is in error by more than 100%. This indicates that if bulk K–Ar or $^{40}\text{Ar}/^{39}\text{Ar}$ techniques had been used on these samples, the differentiated units might have been assigned grossly inaccurate ages, leading to an incorrect interpretation of the local stratigraphy □

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New constraints on early Tertiary palaeoproductivity from carbon isotopes in foraminifera

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ONE of the most pronounced features of marine sections at the Cretaceous/Tertiary (K/T) boundary is the negative excursion in biogenic carbonate $\delta^{13}\text{C}$ in the early Tertiary carbon isotope record¹⁻⁹. The coincidence of this excursion with the elimination of many marine plankton species supports predictions that large-scale reduction of primary productivity in the oceans would disrupt the biological carbon pump, resulting in a diminished surface-to-deep $\delta^{13}\text{C}$ gradient¹⁰ ($\Delta\delta^{13}\text{C}$). Carbonate accumulation rates and other geochemical indices support the idea that the oceans were less productive in the early Palaeocene⁹. Here we present carbon isotope analyses of individual species of benthic and planktonic foraminifera spanning the K/T boundary in ODP Hole 690C (Weddell Sea, Antarctica) which demonstrate that primary productivity following the K/T extinctions may not have diminished to the extent or for the duration indicated by previous isotope studies. The magnitude of the $\delta^{13}\text{C}$ gradient change is dependent on the species analysed, reflecting differing depths of calcification, seasonal contrasts and/or disequilibrium effects. The lack of any change in benthic $\delta^{13}\text{C}$ at the K/T boundary indicates that the Antarctic may not have been a significant source of deep water during the early Palaeocene, contrary to previous suggestion¹¹⁻¹³.

Leg 113 of the Ocean Drilling Program (ODP) drilled at two sites at 65°S on Maud Rise, Antarctica. Together, the two sequences provided a nearly continuous record of biogenic sedimentation through the Cenozoic and late Mesozoic¹⁴. Hole 690C, on the flank of Maud Rise, contains a continuous, latest Cretaceous and Palaeocene section spanning the K/T boundary. The Palaeocene section is composed of foraminiferal-bearing nannofossil ooze with an admixture of clay; the Cretaceous is foraminiferal-bearing calcareous ooze and interbedded chalk^{14,15}. This sequence has also been delineated, using magnetostratigraphy, from chron 29N to chron 32 (ref. 15). The K/T boundary, although bioturbated, is considered to be continuous, although chron 29R seems to be condensed above the K/T boundary¹⁶. The boundary is marked by a distinct iridium anomaly¹⁷ in conjunction with the biostratigraphically placed boundary near the top of chron 29R (refs 18, 19). We performed isotope measurements on species of planktonic and benthic foraminifera, as well as fine-fraction carbonate samples across the K/T boundary. Although none of the planktonic foraminifera analysed is thought to range across the boundary, each was analysed continuously within its range.

A positive $\delta^{13}\text{C}$ shift below the K/T boundary has previously been recognized at equatorial Deep Sea Drilling Program (DSDP) Site 577 in both planktonic and benthic records^{8,9}, and was considered to reflect either a change in the $\delta^{13}\text{C}$ composition of the oceans or a change in deep circulation. It was further suggested that such a shift, if global in scale, might have accompanied reduced carbon flux to the oceans during a rise in sea level during the latest Cretaceous. In Fig. 1, the $\delta^{13}\text{C}$ composition of benthic foraminifera is shown from DSDP Site 577 in the equatorial Pacific⁹, DSDP Site 527 in the south-east Atlantic²⁰ and ODP Holes 689B and 690C from the Antarctic. All of these sections show a similar positive increase in $\delta^{13}\text{C}$ during the 200 kyr immediately preceding the K/T boundary, indicating the global extent of the carbon isotope excursion. The high values continue for 100 kyr into the earliest Palaeocene. Zachos

and Arthur⁸ did not consider the high benthic $\delta^{13}\text{C}$ values of Site 577 in the lowermost Palaeocene to be a continuation of the carbon isotope excursion. Instead, they suggested that early Palaeocene benthic $\delta^{13}\text{C}$ values were high because of reduced primary productivity following the mass-extinction event at the K/T boundary and the subsequent diminished organic carbon flux to deep waters⁸.

In Hole 690C, although the vertical $\delta^{13}\text{C}$ gradient, as measured between the planktonic and benthic foraminifera, was reduced at the boundary, the earliest Palaeocene planktonic foraminiferal $\delta^{13}\text{C}$ values were higher than at any time in the subsequent 2-Myr interval (Fig. 2). The relatively high $\delta^{13}\text{C}$ values of both benthic and planktonic foraminifera in the lowermost Palaeocene reflect the continuation of the positive excursion in the oceanic $\delta^{13}\text{C}$ composition that began 200 kyr before the K/T boundary.

Previous isotope studies of K/T boundary sequences have been carried out on either bulk carbonate (mixture of benthic foraminifera, planktonic foraminifera and calcareous nannofossils), fine-fraction carbonate (primarily calcareous nannofossil and planktonic foraminifera), mixed benthic assemblages, mixed planktonic foraminiferal assemblages or discontinuous measurements of single species¹⁻⁹. These biogenic components each carry a different $\delta^{13}\text{C}$ signature stemming from isotope differences in their respective habitats (surface-, intermediate- or deep-dwellers) or because of different fractionation factors ('vital effects'). The mixture of more than one of these biogenic components in an isotopic sample alters the isotopic signature of interest and makes it difficult, if not impossible, to compare different isotope records. Furthermore, inferences about the vertical distribution of taxa based on isotopic composition and hence, the vertical $\Delta\delta^{13}\text{C}$ gradient, are possible only insofar as the shells of the organisms analysed were secreted in isotopic equilibrium with the sea water in which they lived. However, isotopic disequilibrium is common in marine plankton and varies in magnitude between species and groups²¹⁻³⁰. The isotopic composition of organisms that either photosynthesize or contain photosymbionts, such as modern spinose globigerinid foraminifera, can be particularly susceptible to $\delta^{13}\text{C}$ disequilibrium³¹.

The number of potential factors that can influence the $\delta^{13}\text{C}$ of isotopic samples makes it difficult to assess the accuracy of previous isotope studies in estimating a change in primary productivity at the K/T boundary. Figure 2 shows the $\delta^{13}\text{C}$ values of individual planktonic foraminiferal species from each of the major late Cretaceous groups including *Globigerinelloides multispinatus*, *Abathomphalus mayaroensis*, *Heterohelix globulosa* and concentrated calcareous nannofossils from the Antarctic ODP Hole 690C. *G. multispinatus* has a surface ornamentation similar to that of modern spinose forms. *H. globulosa* is a nonspinose form. The early Palaeocene planktonic species include *Eoglobigerina fringa* from the AP α Zone and *Subbotina pseudobulloides* from the AP1a Zone¹⁹. Neither of these Palaeocene species exhibits pustulose surfaces or displays evidence of spine bases. The benthic foraminifera *Gavelinella beccariiiformis* and *Nuttallides truempyi* were each analysed continuously across the K/T boundary. Each of the foraminiferal groups exhibits a different isotopic composition which reflects either their different depth habitats or possibly is an effect of isotopic disequilibrium. In the Cretaceous, calcareous nannofossils and *G. multispinatus* have the highest $\delta^{13}\text{C}$ values and the lowest $\delta^{18}\text{O}$ values, which indicates that, compared to other species, they either inhabited shallower waters depleted in nutrients and ^{12}C or it reflects isotopic disequilibrium effects. Immediately above the K/T boundary, calcareous nannofossils have values that are lower than those of the planktonic foraminifera *E. fringa* and even lower than benthic foraminifera. These anomalously negative values continued for ~300 kyr after the K/T boundary. It was not until ~66.0 Myr that calcareous nannofossil $\delta^{13}\text{C}$ values once again became higher than plank-

tonic and benthic foraminifera. This same pattern marks many K/T boundary sequences where fine-fraction carbonate and benthic data have been used to estimate surface-to-deep $\Delta\delta^{13}\text{C}$ (ref. 8). Thus, for those sequences that were measured in this way, the $\Delta\delta^{13}\text{C}$ values seem to have been low for 300–500 kyr of the early Palaeocene, the 'Strangelove Ocean' interval. In some deep-sea sequences the $\delta^{13}\text{C}$ inversion is as much as -2.5% (ref. 8) double that of Hole 690C.

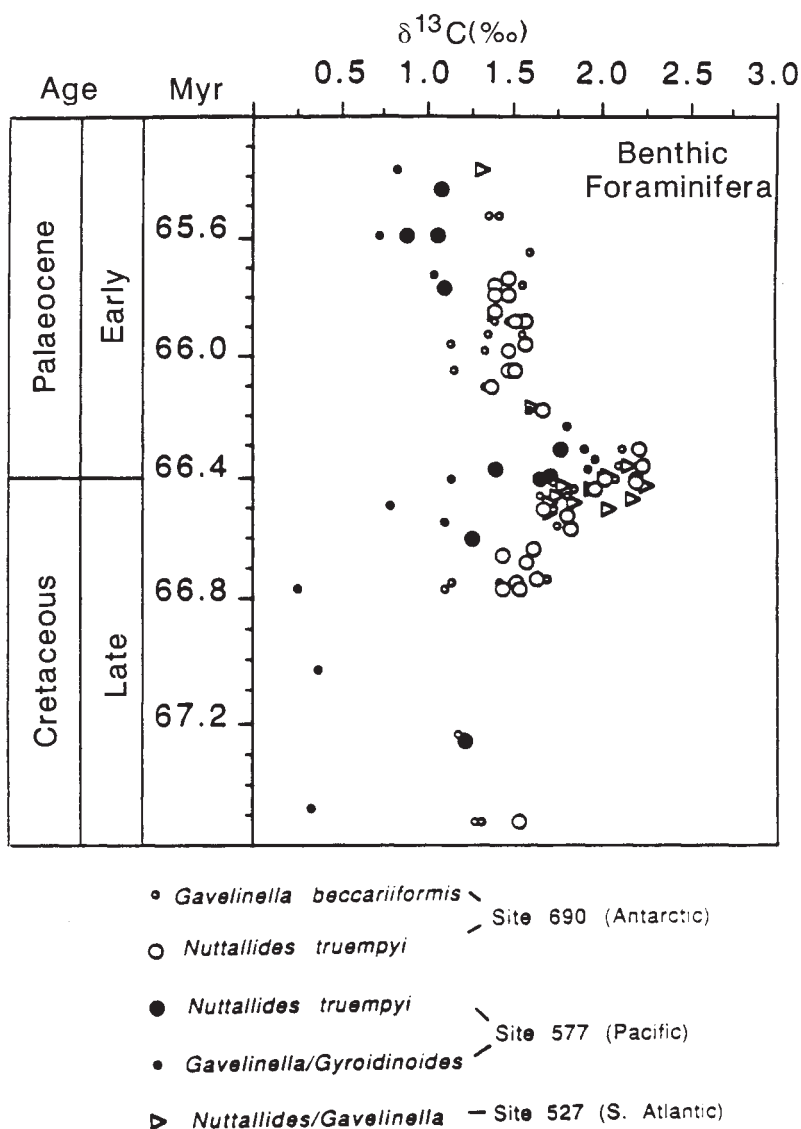
Examination of the planktonic foraminiferal record over the K/T boundary interval in Hole 690C (Fig. 2) results in a somewhat different interpretation. Planktonic foraminiferal $\delta^{13}\text{C}$ and benthic $\delta^{13}\text{C}$ both increase 200 kyr before the K/T boundary. Above the boundary, at the base of Zone AP α , both benthic and planktonic foraminifera $\delta^{13}\text{C}$ values remain high, although the vertical $\delta^{13}\text{C}$ gradient was clearly reduced by $\sim 0.5\%$. Nonetheless, a gradient did exist (0.2%) between planktonic foraminifera and benthic foraminifera immediately following the mass extinctions. Unlike other isotope records, the $\Delta\delta^{13}\text{C}$ gradient between planktonic foraminifera and benthic foraminifera increased during the 100 kyr after this initial reduction.

The calcareous nannofossils, being photoautotrophic, would have inhabited surface waters in association with planktonic foraminifera. Thus, it is difficult to explain their more negative $\delta^{13}\text{C}$ values in the earliest Palaeocene without invoking isotopic disequilibrium during calcification. The nonspinose *E. fringa*

and *S. pseudobulloides* planktonic foraminifera $\delta^{13}\text{C}$ values may also not reflect actual surface-water values but are less likely to have been influenced by photosymbiotic factors if the absence of spines means they did not contain photosymbionts. Furthermore, the $\delta^{13}\text{C}$ values of these early Palaeocene species are similar to Cretaceous values of *H. globulosa*, values that in the Cretaceous, would imply habitats of greater depth (Fig. 2). These results emphasize the problem in determining the extent of productivity changes following the K/T boundary using the $\delta^{13}\text{C}$ of various fossil groups and particularly of mixing with one or more fossil groups.

There are several possible explanations for the apparent disequilibrium fractionation in early Palaeocene calcareous nannofossil $\delta^{13}\text{C}$ values. The growth rate of modern photoautotrophic calcareous nannoplankton is strongly influenced by light intensity and temperature³². Consequently, reduced light intensity and/or temperature variations may influence isotope fractionation during coccolithogenesis. Symbiont-bearing planktonic foraminifera cultured under high light intensity have $\delta^{13}\text{C}$ compositions that are more than 2‰ higher than those grown under dark conditions³³. However, reduced light intensity resulting from a large bolide impact and expulsion of a large dust cloud into the atmosphere³⁴ could not account for the negative excursion in calcareous nannofossil $\delta^{13}\text{C}$ values because the duration of the low-light intensity resulting from such an event would have been too short (several months³⁵ to

FIG. 1 $\delta^{13}\text{C}$ ($[(^{13}\text{C}/^{12}\text{C})_{\text{sample}} / (^{13}\text{C}/^{12}\text{C})_{\text{standard}}] - 1$, PDB standard) of benthic foraminifera from Antarctic Sites 689 and 690, equatorial Pacific Site 577 and South Atlantic Site 527 are reported. Note the positive $\delta^{13}\text{C}$ excursion at all sites that began ~ 200 kyr before the K/T boundary and continued into the earliest Palaeocene.



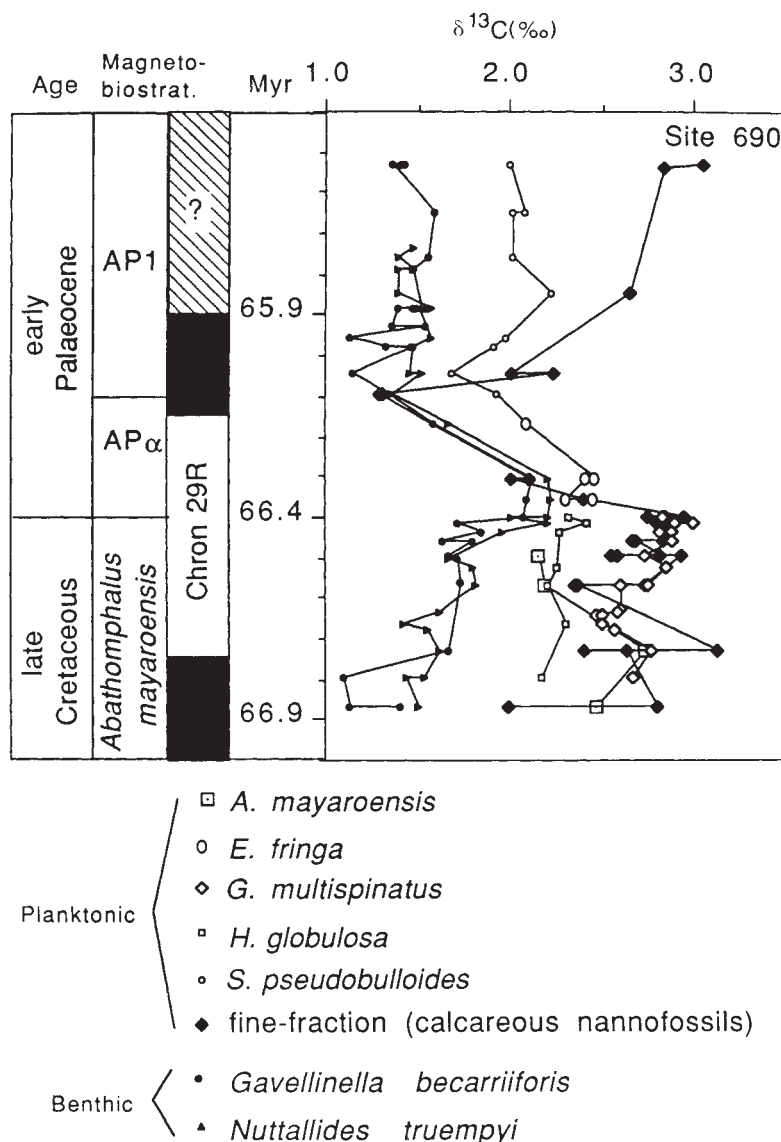
several years³⁴). Culturing studies on modern phytoplankton support the shorter-term (three month) darkness hypothesis³⁶. This is too short to account for the longer-term (~300 kyr) low $\delta^{13}\text{C}$ values in the calcareous nannofossil record.

At any one location in the ocean the $\delta^{13}\text{C}$ of deep waters measured in benthic foraminifera is controlled by two factors: the biological nutrient pump which affects the local surface-to-deep $\delta^{13}\text{C}$ gradient, and the ageing of bottom waters during their journey through the ocean basins. Today, Atlantic deep waters are, on average, 1‰ more positive in $\delta^{13}\text{C}$ than those of the North Pacific³⁷. This reflects the production of nutrient-depleted ($\delta^{13}\text{C}$ -enriched) waters in the North Atlantic, its transport to and mixing with Antarctic waters, and its subsequent incursion, some 1,000 years later into the deep North Pacific.

The $\delta^{13}\text{C}$ composition of benthic foraminifera (*Nuttallides*, *Gavelinella* and *Gyroidinoides*) is plotted in Fig. 1 for sites of equivalent palaeodepth in the south Atlantic²⁰, Antarctic (Hole 690C) and north Pacific⁹ that have well preserved carbonate records and palaeomagnetic age control across the K/T boundary. Below the K/T boundary, a positive gradient existed between the Atlantic and Pacific. Pacific values are generally 1‰ lower than those of the Atlantic, suggesting that circulation during the late Cretaceous reflected nutrient transport from the Atlantic towards the Pacific as in the modern ocean. Immediately following the K/T boundary, that basin-basin $\delta^{13}\text{C}$ gradient was reduced to 0.2–0.3‰. There are several possible reasons for

this. One is that the source of bottom water changed during the 'Strangelove Ocean' event, reversing the direction of transport; or that the rate of oceanic turnover greatly increased. Alternatively, the reduced basin-basin gradient during the 'Strangelove Ocean' might reflect a diminished biological carbon pump resulting from lower primary productivity. Locally, this would have reduced the vertical $\delta^{13}\text{C}$ gradient. It has been suggested that the Antarctic was a source of deep water to the oceans during the late Cretaceous and early Palaeocene^{11–13}. Reduced surface-water productivity in the earliest Palaeocene might be expected to have changed the preformed $^{13}\text{C}/^{12}\text{C}$ ratio of deep waters formed at the surface in the Antarctic. This should be reflected in lower benthic $\delta^{13}\text{C}$ values at Maud Rise. We have shown, however, that the $\delta^{13}\text{C}$ composition of benthic foraminifera immediately following the K/T boundary is essentially the same as those immediately before the boundary (Fig. 1). The preformed $^{13}\text{C}/^{12}\text{C}$ of deep waters formed in the Antarctic did not apparently change significantly following the K/T boundary. Thus some mechanism other than reduced primary productivity (biological carbon pumping) may have contributed to the change in basin-basin $\delta^{13}\text{C}$ gradient of the 'Strangelove Ocean', or the Antarctic was not a significant source of deep waters during the earliest Palaeocene. Detailed and sequential analysis of single planktonic and benthic foraminiferal species across well preserved K/T boundary sequences will allow a better determination of the inferred productivity changes. Interpretations

FIG. 2 $\delta^{13}\text{C}$ of fine-fraction carbonate (primarily calcareous nannofossils), benthic foraminifera and planktonic foraminifera from Site 690, Weddell Sea, Antarctica. Isotope values are reported relative to the PDB standard. Note the reduced planktonic to benthic $\delta^{13}\text{C}$ gradient at the K/T boundary and the lower $\delta^{13}\text{C}$ values of calcareous nannofossils. Calcareous nannofossils values remain lower than benthic and planktonic foraminiferal values until ~66.0 Myr, whereas planktonic foraminiferal values increase relative to benthic values following the K/T boundary.



can be corroborated by analyses of Cd/Ca ratios in benthic and planktonic foraminifera which can provide independent information about nutrient cycling across the K/T boundary³⁸. □

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Possible origin of *n*-alkanes in high-wax crude oils

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LONG-CHAIN *n*-alkanes, with a slight preference for odd carbon number, are major constituents of high-wax crude oils. It is generally believed that these *n*-alkanes form by defunctionalization of (loss of functional groups from) the major components of higher-plant cuticular waxes¹. Recently however, another cuticular component—an insoluble, non-hydrolysable highly aliphatic biopolymer^{2,3}—was proposed as a possible biological precursor of these *n*-alkanes³. Here we study *n*-alkane distributions in high-wax crude oils through a series of artificial maturation experiments performed on one such biopolymer, isolated from the leaf cuticle of the extant vascular plant *Agave americana* L. At relatively low temperatures, the homologous series of *n*-alkanes generated exhibits an internal distribution pattern that is indistinguishable

from that of *n*-alkanes in some naturally occurring high-wax crude oils. Similar types of resistant biopolymers present in algal cell walls might be a major source of *n*-alkanes in non-waxy crude oils.

Like other major constituents of crude oils, the *n*-alkanes are thought to be derived mainly from the thermal degradation of kerogen, the macromolecular organic material in sediments that is insoluble in common organic solvents^{1,4}. Despite numerous studies in recent decades, many uncertainties remain concerning the exact genetic relationships between these *n*-alkanes, kerogen and the putative, biological kerogen precursors. Knowledge of precursor-product relationships is of fundamental interest in organic and petroleum geochemistry because it will provide us with a better insight into the mechanisms of oil genesis and maturation.

Recently, the presence of insoluble, non-hydrolysable highly aliphatic biopolymers in cell walls of several fossil and extant algae and in protective outer layers of higher plants was demonstrated^{2,3,5–7}. Furthermore, it has been demonstrated by combined chemical and microscopy studies that, by a mechanism of selective preservation and consequently selective enrichment, these types of highly aliphatic biopolymers are probably the main precursors of aliphatic moieties in humic substances⁸ and kerogens^{5–7}. Such a mode of kerogen formation opposes the popular neogenesis model in which kerogen is considered to result from abiological random polymerization of monomers derived from the biodegradation of biopolymers, such as polysaccharides and proteins^{1,4}.

Because of their highly aliphatic nature and as a consequence of their selective preservation and consequently selective enrichment during diagenesis, these biopolymers have to be given serious consideration as biological precursors of *n*-alkanes in crude oils^{3,7}. To test this we have performed a series of artificial maturation experiments on one such insoluble, non-hydrolysable highly aliphatic biopolymer, isolated from the leaf cuticle of the extant *A. americana*. This cuticle was chosen as it is considered to be representative of many extant higher plants (refs 2, 3 and Tegelaar *et al.*, manuscript in preparation). The relevance of this type of experiment might be criticized because of its completely different time-temperature relationship as compared with geological processes. The results of several artificial maturation experiments performed on different kerogen types using variable conditions have shown, however, that the processes of petroleum generation and maturation can be mimicked^{9–12}.

Isolation of the highly aliphatic biopolymer was achieved by sequential removal of the other cuticular constituents—waxes, cutin and polysaccharides¹³. Between 10 and 20 mg of the freeze-dried isolate was put into small gold tubes and sealed either in the presence or absence of water. The sealed tubes were placed in externally heated cold-seal pressure vessels¹⁴. To minimize the risk of rupture during the heating experiments the tubes were subjected to an external pressure of 3×10^7 Pa. The duration of the experiments varied from 1 to 16 weeks and temperatures varied from 150 to 400 °C. After the experiments the tubes were cooled, opened and weighed, the weight loss of the tubes being a measure of the volatile products generated. The contents of the tubes were subjected to flash heating to 358 °C and the evaporated products obtained (the 'thermal extract') were analysed on-line by gas chromatography to minimize losses of low-molecular-weight materials¹⁵.

Thermal degradation of the highly aliphatic biopolymer in the temperature range of 275–375 °C resulted in the almost exclusive generation of *n*-alkanes. Representative gas chromatograms, showing characteristic alkane distribution patterns of the thermal extracts, are shown in Fig. 1. The duration of the experiments, within the time limits investigated, and the addition of water do not have a significant qualitative or quantitative effect on the compounds generated. In the temperature range of 275–325 °C the gas chromatograms of the thermal extract are characterized by a bimodally distributed homologous series of

n-alkanes, showing maxima at C_{17} and C_{29} . The longer chain *n*-alkanes have the highest relative abundance and exhibit a noticeable odd-carbon-number preference (CPI ≈ 1.2 (ref. 16); Fig. 1a). A minor series of methyl-branched alkanes is observed. The distribution of these artificially generated hydrocarbons is virtually indistinguishable from that of the saturated-hydrocarbon fraction of certain naturally occurring high-wax crude oils, as exemplified by the gas chromatogram of an Indonesian crude oil (Fig. 2). Rupture of relatively labile bonds within the tertiary structure of the highly aliphatic biopolymer probably gives rise to the generation of the specific distribution in the *n*-alkanes¹³. These findings are in contrast with the generally accepted rationale for the *n*-alkane distribution pattern in high-wax crude oils, which is that of the defunctionalization of the major components of higher plant waxes¹. In addition, past thermolysis experiments performed on wax compounds, such as fatty acids, did not generate the distribution patterns of *n*-alkanes observed in high-wax crude oils¹⁷.

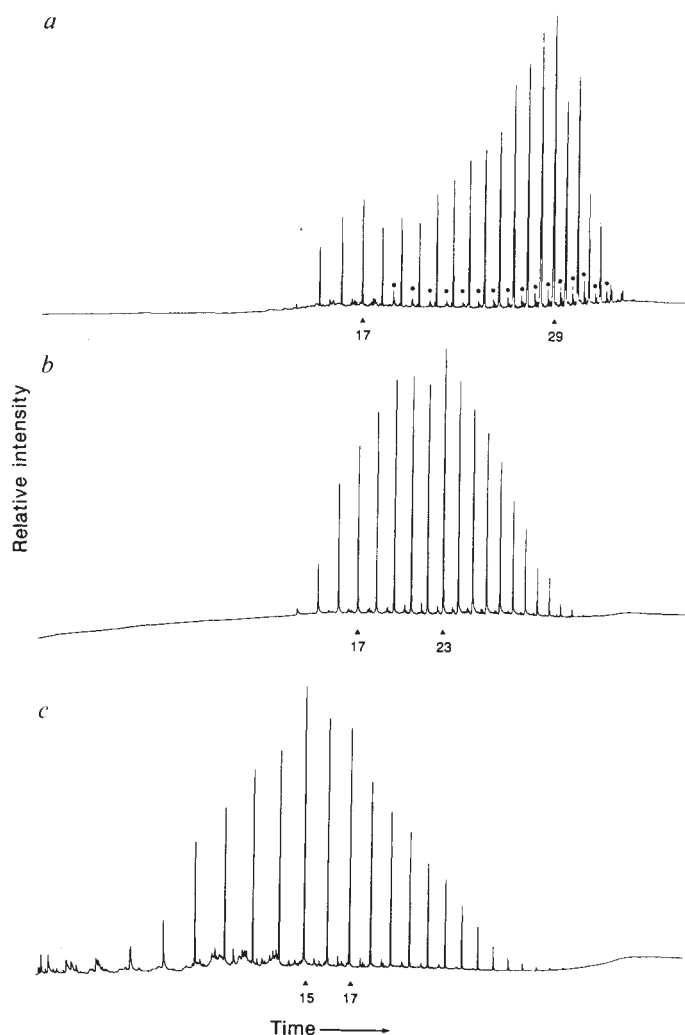


FIG. 1 Gas chromatograms of the thermal extracts of the artificially matured isolated highly aliphatic biopolymer of *A. americana* at a, 325 °C, 4 weeks, b, 350 °C, 4 weeks, c, 375 °C, 4 weeks. The gas chromatographic analyses were performed as previously described¹³. Instrumental conditions: Curie-point temperature 358 °C; gas chromatographic separation was achieved on a fused-silica column (length=25 m; inner diameter=0.31 mm) coated with chemically bonded CP-Sil 5 (film thickness=0.4 μ m); temperature programme of the gas chromatograph oven: 0–320 °C at 3 °C min⁻¹, held at 320 °C for 20 min. The numbers indicate the chain length of the *n*-alkanes; solid circles denote methyl-branched alkanes.

With increasing temperature there is a general shift towards the genesis of lower-molecular-weight hydrocarbons (Fig. 1b), ultimately resulting in a smooth and regularly decreasing *n*-alkane distribution in the thermal extract of the sample matured at 375 °C (Fig. 1c). The observed trend agrees with results obtained for natural and artificial maturation series of the Mahakam delta coals¹¹; our results, however, are derived not from kerogens but from biological kerogen precursors. The distinct increase in the production of low-molecular-weight material is clearly reflected in the relative weight loss of the sample. In the temperature range 150–350 °C, conversion of starting material into gaseous products is constant, ~ 20 wt%. Using mass spectrometry we found that CO₂ accounts for 90% of the generated gases for the sample heated at 325 °C, whereas only minor amounts of low-molecular-weight hydrocarbons such as methane, ethane and propane were detected. These unusually high amounts of CO₂ are consistent with results from artificial maturation experiments performed on a Tertiary brown coal¹⁰. Here the CO₂ may originate from the thermal degradation of a polysaccharide moiety present in the isolate^{13,18}. The significant decrease in weight ($\sim 50\%$) for the sample heated at 375 °C probably indicates the beginning of secondary thermal cracking, resulting in the enhanced generation of low-molecular-weight volatile hydrocarbons. At 400 °C the amounts of low-molecular-weight hydrocarbons generated caused tube rupture, despite the external pressure of 3×10^7 Pa.

Our results confirm the suggested high hydrocarbon generation potential of the insoluble, non-hydrolysable highly aliphatic biopolymer present in higher plant cuticles. But highly concentrated accumulations of morphologically recognizable fossilized remains of higher plant cuticles (cutinite) only occur in unusual circumstances, such as those that produced the Indiana Paper Coal¹⁹. This does not rule out the fact that in some cases amorphous kerogen can be derived from microscopic non-recognizable residual cuticular material. Indeed, it has been established that especially the 'matrix liptinite', consisting of finely comminuted and amorphous materials of possible

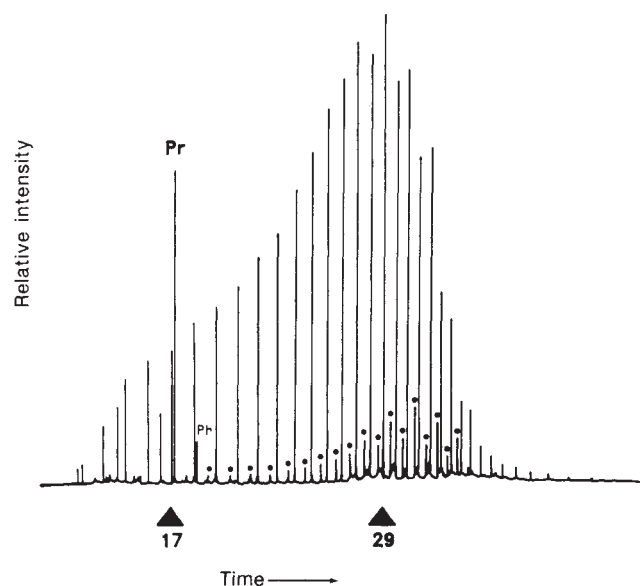


FIG. 2 Gas chromatogram of the saturated-hydrocarbon fraction of a high-wax crude oil from Indonesia. The gas chromatographic analysis was performed on a HP 5890. Separation was achieved on a 25-m fused-silica column (inner diameter=0.2 mm) coated with SE-54 (film thickness=0.33 μ m). Temperature programme of the gas chromatograph oven: 100–200 °C at 5 °C min⁻¹ and from 200 to 325 °C at 6 °C min⁻¹, held at 325 °C for 35 min. The numbers indicate the chain length of the *n*-alkanes; solid circles denote methyl-branched alkanes. Pristane (Pr) and phytane (Ph) are not generated from the highly aliphatic biopolymer but originate from other sources^{23,24}.

cuticular or even algal origin, have the potential to generate high-wax crude oils²⁰. In addition, it is anticipated that similar insoluble, non-hydrolysable highly aliphatic types of biopolymers present in algal cell walls^{5-7,21,22} exhibit a similar behaviour towards catagenesis as that described for the highly aliphatic biopolymer in higher plant cuticles and that the algal-derived biopolymers might be major precursors of the *n*-alkanes in non-waxy crude oils. Artificial maturation experiments are being performed to study the catagenetic behaviour of this type of algal-derived resistant biopolymers. □

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Role of hydrothermal precipitates in the geochemical cycling of vanadium

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THE input of high-temperature fluids to the ocean along sea-floor spreading centres plays an important part in controlling the composition of sea water¹⁻³. Available data¹⁻³ show that hydrothermal systems are major sources of lithium, manganese, rubidium, iron and silicon and sinks for magnesium and sulphur. Furthermore, reactive precipitates formed in vent systems or emerging plumes⁴⁻⁶ scavenge elements from sea water, thereby also influencing the composition of the sea water. Our work on the Mid-Atlantic Ridge, combined with data from the Pacific Ocean, shows that scavenging by vent-derived iron oxides helps to control the concentration and cycling of vanadium in the ocean. We estimate that 10-60% of the riverine input of vanadium to the ocean is removed from sea water by this mechanism and that such a process is also important for other elements.

The significance of trace-element scavenging by iron oxides in the marine environment has been considered for many years^{7,8}. In 1937, Goldschmidt⁷ stated that 'poisonous elements'

such as arsenic, bismuth, antimony, selenium and lead could be removed from aqueous solution by "adsorption on freshly precipitated hydroxides of iron". Laboratory experiments⁸ showed >95% efficiency for vanadium uptake by iron oxides but adsorption was ruled out as an important mechanism for vanadium removal from the oceans because clays, apatite, manganese oxides and organic matter were inefficient scavengers of vanadium. Recent discoveries of abundant hydrous iron oxides in hydrothermal plumes^{5,6,9,10} have rekindled interest in this removal mechanism. Data from the Atlantic and Pacific Oceans indicate that such scavenging has a significant role in the geochemical cycling of vanadium and possibly other elements. Concurrent work¹¹ shows that scavenging by vent-derived iron oxides is also important in the phosphorus cycle.

In dispersed hydrothermal plumes on the Mid-Atlantic Ridge (MAR) at 26° N, precipitates are rich in amorphous iron oxides (10-35% Fe) at suspended particle levels of 20-400 µg l⁻¹ (refs 10, 12). The weight per cent of iron in suspended particles decreases away from the vent source as hydrothermal precipitates mix with ambient suspended matter from the deep North Atlantic. Concentrations of vanadium in plume particles correlate well with those for iron (Fig. 1). This observation is consistent with scavenging of vanadium from sea water by iron oxides, as we substantiate below. High-temperature vent solutions are probably not the source of this vanadium because laboratory studies of sea water/basalt interactions show no vanadium release¹³ and because concentrations in endmember fluids are generally near seawater values^{14,15}.

Analyses of water-column samples also provided evidence for vanadium scavenging. Two of our fresh, near-vent samples, collected from depths of ~3,670 m and with endmember fluid dilutions¹⁶ of ~3,000, had dissolved vanadium values of 24.3 and 26.1 nmol kg⁻¹. These concentrations are significantly lower than background seawater levels of 30.0 ± 0.5 nmol kg⁻¹. However, vanadium was not adsorbed on to the walls of the sample bottle. Most of the 'missing' 4-6 nmol V kg⁻¹ in these samples were taken up by iron oxides present in the water sample during the four hours before filtration. These samples attained particulate vanadium concentrations of 8.7 and 8.8 nmol kg⁻¹ and thereby 'equilibrated' at the V/Fe ratio observed for other plume particles (Fig. 1). Considering the sizeable dilution of vent fluid and the large negative anomaly for dissolved vanadium (4-6 nmol kg⁻¹) relative to the concentrations of particulate vanadium (~8.8 nmol kg⁻¹) in the two samples, it is unlikely that much of the dissolved vanadium removal occurred in the water column. This conclusion is supported by data for dissolved phosphorus in a vent plume from the Pacific¹¹ where a maximum

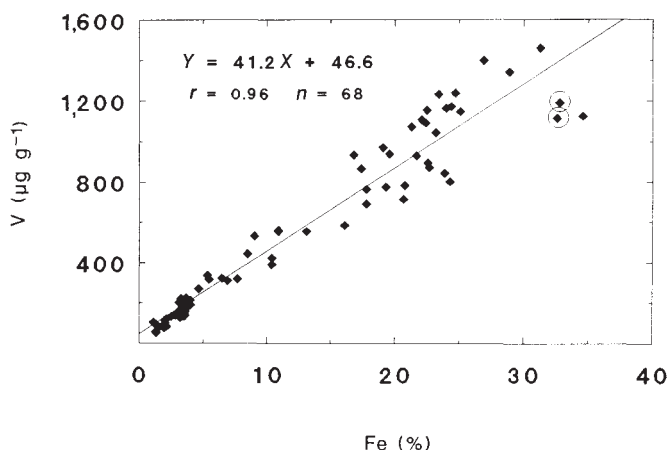


FIG. 1 Graph showing concentrations of iron plotted against vanadium for suspended matter samples collected in a vent plume on the Mid-Atlantic Ridge at 26° N (ref. 10). The open circles identify samples with a negative dissolved vanadium anomaly.

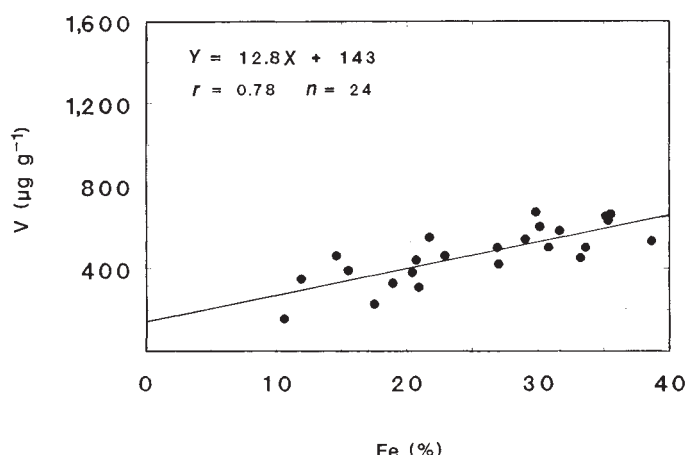


FIG. 2 Graph showing concentrations of iron plotted against vanadium for sediments from the Mid-Atlantic Ridge at 26°N (ref. 18). Data for sediment layers containing sulphide minerals and >90% hydrothermally derived material (as defined in ref. 18) are omitted because sulphides and secondary iron oxides, abundant in the >90% hydrothermal layers, have a low vanadium content.

negative anomaly of $\sim 60 \text{ nmol kg}^{-1}$ or $\sim 2\%$ of the total dissolved phosphorus was found. Thus we observed an interruption of the normal scavenging process for dissolved vanadium from an essentially infinite reservoir in the MAR rift valley by containment in a vanadium-limited, 30-l sampler. At vent-fluid dilutions over 3,000, vanadium uptake by iron oxides was completed *in situ* and no detectable negative anomaly for dissolved vanadium was observed. These trends indicate that scavenging of vanadium is rapid and controlled by a predictable V/Fe affinity. In laboratory experiments¹⁷ using synthetic sea water (35‰ salinity) and amorphous ferric oxyhydroxide (2 mM), 80% of the available dissolved vanadium (200 μM) was scavenged within two minutes. Although the iron and vanadium levels were high in these experiments, they demonstrate the rapid uptake of vanadium by iron oxides.

Further evidence for vanadium scavenging was found in a metalliferous sediment core collected at 26°N on the MAR. Concentrations of vanadium in the sediments ranged from 140 to 1,000 $\mu\text{g g}^{-1}$ (CaCO_3 -free)¹⁸. Iron values were approximately uniform at 40% (CaCO_3 -free) throughout this core with no significant linear relationship between vanadium and iron¹⁸. Low vanadium and high iron concentrations in these sediments were found at intervals containing sulphide minerals, indicating that vanadium uptake did not occur on or with sulphide phases. Sulphides from 26°N on the MAR and along the East Pacific Rise (EPR) contain $<100 \mu\text{g V g}^{-1}$ (refs 6, 19). When data for sediment intervals containing a significant sulphur component were excluded, the V/Fe relationship improved (Fig. 2). The lower slope and greater scatter for the sediment V/Fe

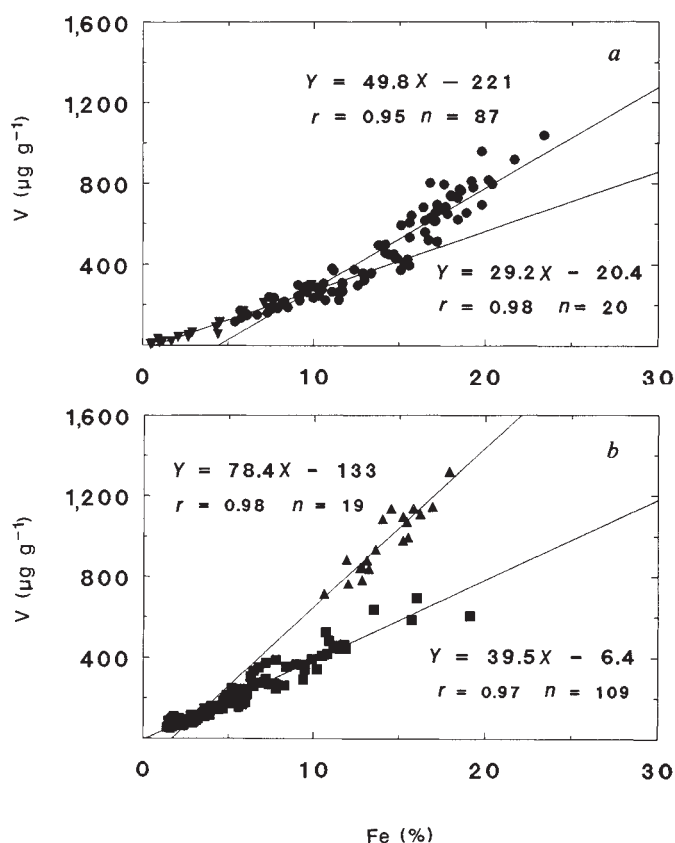


FIG. 3 Graphs showing concentrations of iron plotted against vanadium for sediments from the East Pacific Rise. a, Data from ref. 20 [▼]; ref. 22 [●]. b, ref. 22, DSDP [■]; ref. 21 [▲]. Linear regression equations and complementary data correspond to adjacent lines.

plot (Fig. 2) relative to that for the suspended particles (Fig. 1) can be attributed to the following: (1) differences in the initial amount of vanadium taken up by primary iron oxides as a function of grain size, rate of precipitation and concentrations of competing species; (2) the presence of variable amounts of secondary iron oxides formed from vanadium-poor sulphides; and (3) diagenetic alteration of primary iron oxides over time leading to variable release of vanadium and iron.

Observed V/Fe trends in hydrothermal sediments and suspended matter from the Atlantic can be extended to sediments recovered from the East Pacific Rise²⁰⁻²² (Fig. 3). Two data sets are included on each graph (Fig. 3a, b) and separate slopes have been determined for each. Three of the four data sets have an average V/Fe ratio of 4×10^{-3} , comparable with suspended particles from the Atlantic, whereas the remaining sediments²¹ (Fig. 3b) have a V/Fe ratio of 7.8×10^{-3} . At present, variations

TABLE 1 Data calculation showing the role of hydrothermal iron oxides in the global vanadium cycle

	Range	Best estimate
Hydrothermal flux (refs 2, 3, 24-27, 33)	$0.3-1.5 \times 10^{14} \text{ kg yr}^{-1}$	$0.7 \times 10^{14} \text{ kg yr}^{-1}$
High-temperature hydrothermal endmember Fe concentration (refs 3, 28-31)	$0.5-18 \times 10^{-3} \text{ mol kg}^{-1}$	$\times 1.0 \times 10^{-3} \text{ mol kg}^{-1}$
		$= 0.7 \times 10^{11} \text{ mol Fe yr}^{-1}$
Fraction of Fe precipitating as hydrous oxides (ref. 6)	$50 \pm 30\%$	$\times 0.50$
		$= 0.35 \times 10^{11} \text{ mol Fe yr}^{-1}$
V/Fe ratio (refs 10, 20-22)	$2-8 \times 10^{-3}$	$\times 4 \times 10^{-3}$
		$= 1.4 \times 10^8 \text{ mol V yr}^{-1}$
Riverine V flux ²³		$5.0 \times 10^8 \text{ mol V yr}^{-1}$

in the V/Fe relationship for these data can only be ascribed to a combination of the effects listed above. Elevated vanadium concentrations and the strong V/Fe trend found for sediments recovered during Leg 92 of the Deep Sea Drilling Project²² (Fig. 3b) date back to the Oligocene (26–37 Myr BP (before present)). Collectively these data suggest that scavenging of vanadium by hydrothermal iron oxides has widespread geographical and temporal significance.

How important is vanadium scavenging by vent-derived iron oxides to the vanadium cycle? At 100,000 yr (ref. 23), the residence time of vanadium in sea water is relatively long and little information is available on removal mechanisms. Previous geochemical balances for vanadium assumed no vanadium uptake from sea water by hydrothermal precipitates, relating essentially all of the vanadium removal to 'authigenic subtraction' in the upper water column¹⁴. We believe that available data on global venting provide sufficient information to show that scavenging of vanadium by hydrothermal iron oxides is an important 'authigenic' removal mechanism that needs to be factored into the global vanadium cycle.

To determine the magnitude of vanadium removal by hydrothermal iron oxides, we need to know the flux of high-temperature fluid from ridge vents, the iron content of the emerging fluid, the fraction of iron oxides formed and the V/Fe ratio of the oxide phase. Estimates of hydrothermal fluxes from axial locations are complicated by a variety of factors; however, the range of estimates is presently constrained to within a factor of 5–6. Palmer and Edmond²⁴, in balancing the strontium isotope budget for the ocean, recently calculated the flux of sea water through high-temperature hydrothermal systems to be $1.2 \pm 0.3 \times 10^{14}$ kg yr⁻¹. The upper limit of this estimate is consistent with that in ref. 2. By contrast to these values, high-temperature fluxes of $\sim 0.3 \times 10^{14}$ kg yr⁻¹ have been estimated^{25,26}. Based on balancing the magnesium budget for the ocean, values of $0.6\text{--}0.8 \times 10^{14}$ kg yr⁻¹ are reported^{24,27}. Here we will use a range of $0.3\text{--}1.5 \times 10^{14}$ kg yr⁻¹ with a best value of 0.7×10^{14} kg yr⁻¹.

The iron content of high-temperature fluids is controlled by temperature as well as chloride and sulphide content^{3,28}. Observed endmember iron concentrations range from 0.5 to 18 mM at temperatures of 270–350 °C (refs 28–31). These values are consistent with model calculations³² for exit temperatures of 250–350 °C. More information is needed on the global distribution of vent temperatures; however, based on available data we have chosen 1 mM iron as a best value.

The fraction of primary iron oxide formed in the vent plume is reported⁶ as $50 \pm 30\%$. Some variability in the V/Fe ratio is observed (Figs 1–3); however, a value of 4×10^{-3} is our best estimate at this time. Using the assembled data, we calculate that $0.6\text{--}3 \times 10^8$ mol V yr⁻¹ can be removed from sea water with a best estimate of 1.4×10^8 mol V yr⁻¹ (Table 1). Such removal accounts for 10–60% of the dissolved vanadium input by rivers (Table 1). Our best estimate is that $\sim 25\%$ of the riverine influx of vanadium is removed through scavenging by hydrothermal iron oxides. Further work in our laboratory and other ongoing studies^{11,15,33} suggest that hydrothermal precipitates scavenge phosphorus, arsenic, chromium and possibly other elements. Feely *et al.*³⁴ estimate that 10–30% of the global phosphorus removal occurs through scavenging by vent-derived iron oxides. Preliminary data for the MAR and sediment data from the Pacific^{21,22} show a strong As/Fe relationship with an As/Fe ratio of $\sim 1 \times 10^{-3}$ and particulate arsenic values as high as $600 \mu\text{g g}^{-1}$. Thus, the abundant iron oxides in vent plumes have emerged as a potentially significant component of the geochemical cycle. □

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Heat-flow variations correlated with buried basement topography on the Juan de Fuca Ridge flank

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SYSTEMATIC heat-flow variations are commonly observed on sedimented flanks of mid-ocean ridges. These variations have been attributed to the convective circulation of pore fluids within the oceanic crust, and have been used to estimate the scale and pattern of this circulation. Here we report similar systematic variations observed in a detailed heat-flow and seismic-reflection survey over sediment-covered 3-Myr-old sea floor on the eastern flank of the Juan de Fuca Ridge. We find that heat-flow peaks are located above buried basement ridges and adjacent to two minor basement outcrops; heat-flow lows are found where thick sediments fill basement valleys. The simple inverse relationship between heat flow and sediment thickness defined by the data indicates that hydrothermal circulation sealed within the permeable portion of the oceanic crust is vigorous enough to maintain approximately isothermal (± 10 K) conditions at the sediment/crust interface. As no systematic variations in estimated basement temperatures are observed, it seems that at this location the heat-flow variations result only from sediment thickness variations, and do not reflect a particular convective pattern.

The existence of pervasive convection of sea water through oceanic crust was postulated originally to account for locally high heat-flow variability and the observed discrepancy between measured heat flow and that predicted by thermal models of cooling oceanic lithosphere^{1–4}. At mid-ocean ridges, hydrothermal circulation is manifested in high-temperature (350 °C) hydrothermal vents where locally high rates of heat and chemical

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flux occur⁵. In ridge-flank settings, circulation continues as the crust ages and cools, but at lower temperatures and flow rates. In the absence of continuous^{6,7} or adequately thick⁸ sediment cover, significant advective heat exchange occurs through the sea floor. Under conditions of complete burial, hydrothermal circulation continues within the crust, but advective exchange of fluids through the sea floor is restricted and the average heat flow for a region approaches that predicted for cooling lithosphere. Eventually (after many tens of millions of years) the permeability of the oceanic crust may be reduced sufficiently by alteration and mineral deposition to prevent pore-fluid convection.

Patterns of crustal hydrothermal convection in young sedimented ridge-flank settings have been inferred from measured patterns of seafloor heat-flow variability^{4,9,10}, and modelled using numerical and laboratory simulations of coupled fluid flow and heat transfer through porous media^{1,9,11-14}. Systematic high and low heat-flow anomalies are commonly observed that strike parallel to structural grain^{15,16}. The cross-strike heat-flow variations commonly have a wavelength of 6–10 km, and are often correlated with seafloor topography, with minima near topographic lows and maxima near scarps or topographic highs. These variations have been interpreted to be related directly to cellular convection of pore fluids within the oceanic crust. If,

as is commonly assumed, the convection cells have an aspect ratio near unity even in the presence of topography¹⁷, then the hydrothermal convection extends to depths of 3 to 5 km into the oceanic crust. Although determinations of permeability in oceanic crust are sparse, they indicate that the general permeability structure is strongly layered; the upper few hundred metres of igneous crust is several orders of magnitude more permeable than either the deeper crust or the overlying sediments¹⁸⁻²³. If this is generally true, then convection cells cannot be of both several kilometre dimension and unit aspect ratio. It has been suggested that localized hydrothermal recharge or discharge, or the thermal perturbations caused by basement topography^{9,24} strongly force the regional-scale pattern of hydrothermal circulation and cause high-aspect-ratio circulation or convection cells to develop. If this is the case, then the wavelength of the heat-flow anomalies may not provide information about the depth to which circulation penetrates. Our observations indicate that, in some cases, the wavelength of systematic heat-flow variations may not even provide information about the horizontal scale of convection cells.

Until recently, few surveys provided the combination of detailed seismic imaging of the sediment–basement interface and coincident closely spaced heat-flow measurements required to characterize properly the nature of hydrothermal convection

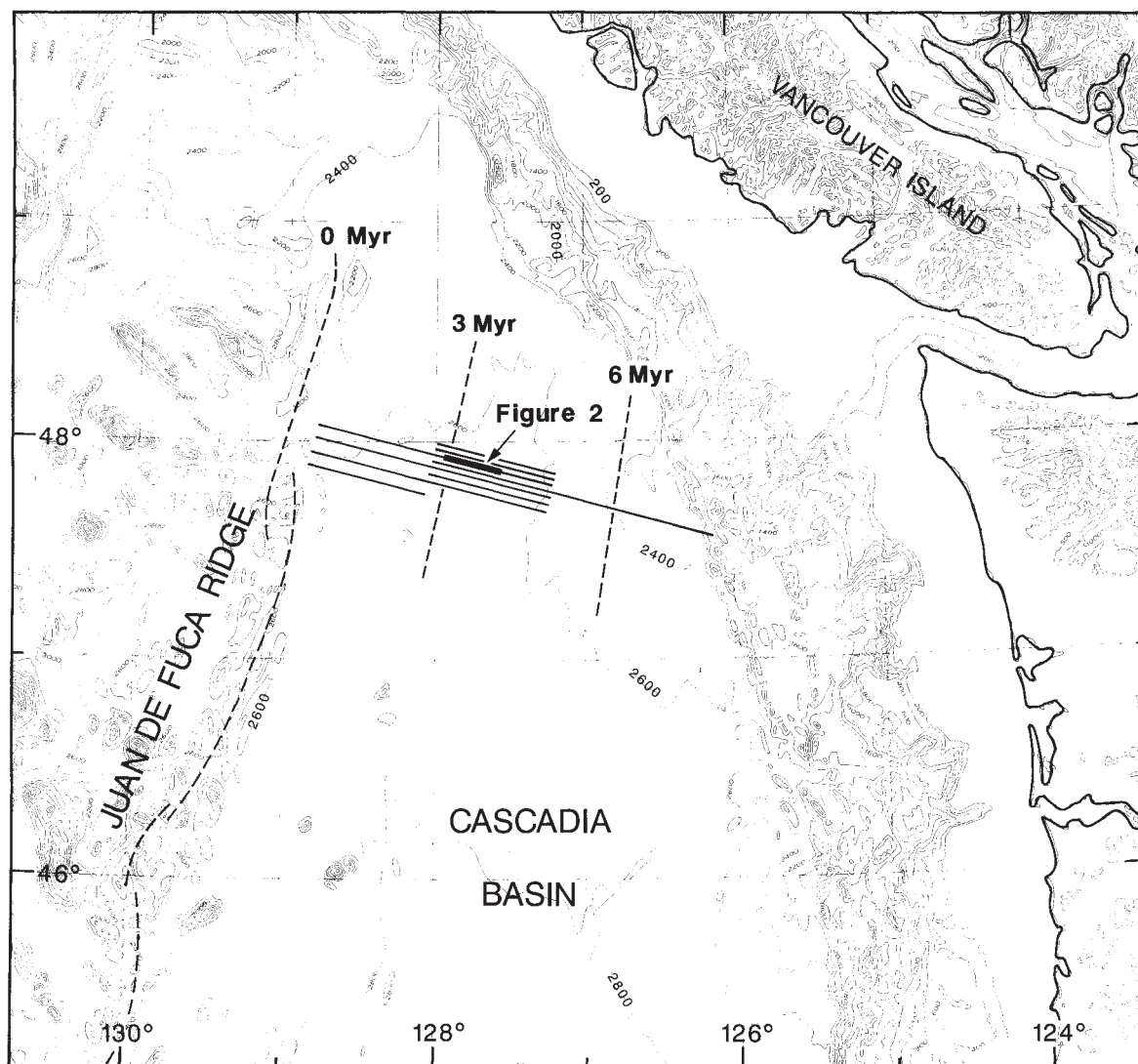


FIG. 1 Location map for the 1988 'Flankflux' heat-flow and seismic surveys in Cascadia Basin on the eastern flank of the Juan de Fuca Ridge. Isochrons for the oceanic crust are shown by dotted lines. Thin solid lines show the

location of the single-channel seismic profiles along which we gathered the heat-flow data used in Fig. 3. The position of the seismic and heat-flow profile shown in Fig. 2 is highlighted.

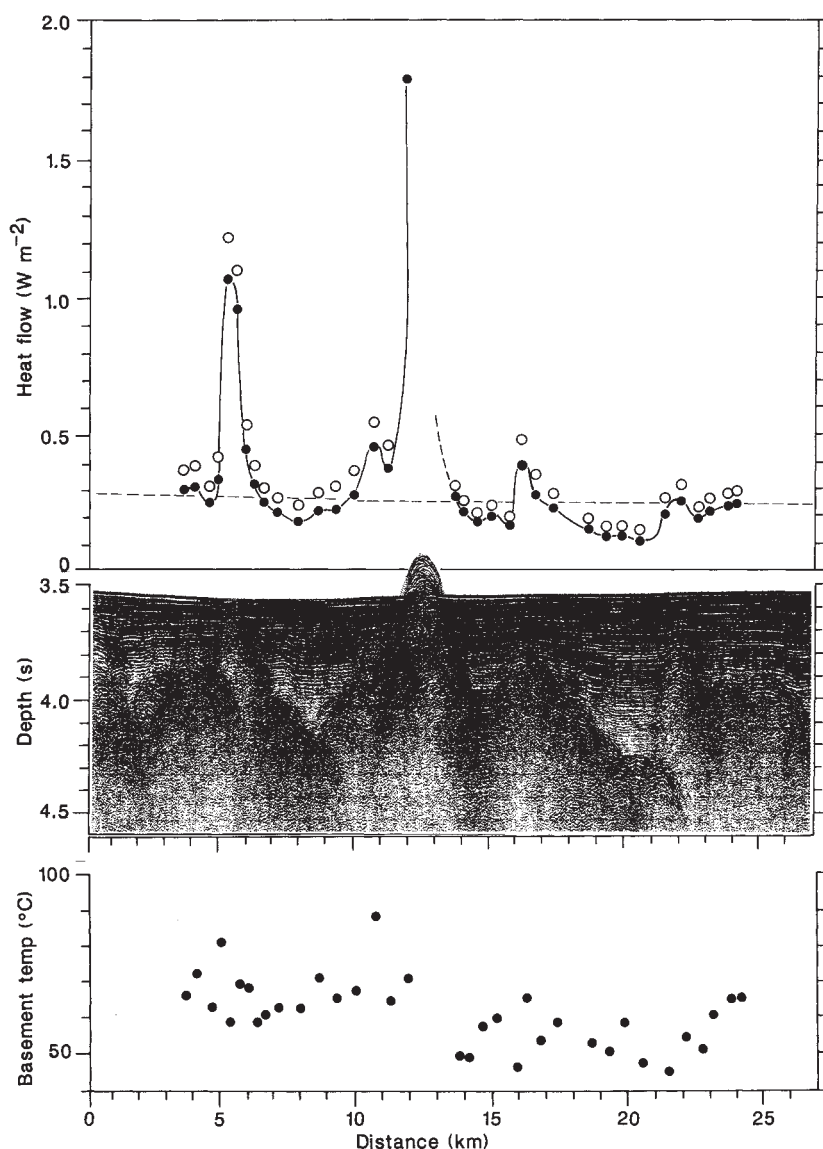
in ridge-flank environments. In the autumn of 1988, during a cruise of the CHS *Parizeau* to the eastern flank of the Juan de Fuca Ridge, we collected a data set that provides this combination. We collected roughly 1,400 km of high-quality single-channel seismic profiles along the closely spaced lines shown in Fig. 1. In the western part of the survey area, the basement surface is remarkably smooth (<100 m of local relief) and buried by a uniform 300-m thickness of sediment; we plan a detailed heat-flow study to explore the nature of hydrothermal convection in the absence of significant basement topography or sediment thickness variations for this area in 1990. East of a longitude of about $127^{\circ}50'$ W, local basement relief is much higher, up to 800 m, and strongly two dimensional. Turbidite sediments from the continental margin of western Canada bury all of the basement relief except for two small conical volcanic edifices situated at the crests of two adjacent basement ridges, roughly 10 km apart. This eastern part of the area proved to be ideal for studying the effects of sediment thickness variations, basement topography and basement outcrops on hydrothermal circulation. Towards this end, we made 170 heat-flow determinations along nine multipenetration heat-flow profiles crossing the strike of basement structure and radiating away from the basement outcrops. We determined heat flow as the interval product of

temperature gradients and *in situ* thermal-conductivity values obtained with a 2.5-m long, 11-thermistor violin-bow probe²⁵. Navigational control was provided by GPS (global positioning system satellite array) and GPS-corrected Loran-C. Careful station keeping and the use of a heavy instrument allowed us to determine positions of the heat-flow measurements along the seismic lines to within about 100 m.

A heat-flow profile running across one of the two isolated outcrops and crossing three other sediment-covered ridges is shown in Fig. 2. Heat flow along this profile varies from 125 to $1,800 \text{ mW m}^{-2}$. The common occurrence of extreme values close to the outcrops indicates that some heat is lost by advection of fluids through these 'permeable penetrators' of the sea floor. With the exception of the extreme heat-flow peaks, however, values along this profile conform, on average, to the level of heat flow predicted for a conductively cooling oceanic lithosphere. Thus it seems that the amount of heat lost by advection through these outcrops and through the sediments that bury the remaining basement relief is small compared with the total heat loss in the area.

The most striking characteristics of this profile are the four well defined heat-flow peaks spaced roughly 6 km apart that are clearly correlated with buried basement relief, and perhaps more

FIG. 2 Coincident heat-flow and single-channel seismic profiles from the sedimented eastern flank of the Juan de Fuca Ridge (see Fig. 1 for location). Solid circles represent raw heat-flow determinations; open circles represent heat flow corrected for the thermal effects of sedimentation. Dashed line indicates the theoretical heat flow expected from lithosphere of appropriate age. We estimated temperatures at the sediment-basement boundary from the uncorrected heat-flow values and the depths to basement estimated from the seismic profile.



importantly, with sediment thickness variations. The estimated thermal effects of sedimentation in this environment (calculations provided by S. Willett) are significant (Fig. 2) but do not account for the anomalies. Similarly, computer simulation shows that the focusing of conductive heat flow through areas of thin sediment cover is insufficient to explain our results; non-conductive heat transfer within the crust beneath the sediments seems to be required.

Similar scales of heat-flow variability, and a correlation between heat flow and basement topography have been observed by other workers in regions of sediment-draped basement^{4,9,15,16}. Those observations led to the hypothesis that basement topography and sediment thickness variations strongly control the pattern and scale of circulation. Model simulations of convection at near- or sub-critical Rayleigh numbers²⁴ indicate that locations of downwelling and hydrothermal recharge are forced to occur beneath valleys where isotherms in the crust are depressed following the topography; upwelling and discharge through the sediment section occur preferentially above basement ridges where isotherms are elevated, and where sediments may be thinner and the hydraulic impedance of the sediment 'seal' diminished. In the simulations, convection cells of extremely high aspect ratio develop, with horizontal cell dimensions commensurate with topography (several kilometres) and with most of the fluid flow confined to the upper, most permeable part of the crust (few hundred metres).

By contrast to the situation in areas of sediment-draped topography, the basement relief in our study area is almost completely buried by flat-lying turbidite sediments. Sediment thickness variations are large (of the same magnitude as basement relief) and, under conductive or near-critical convective conditions, the deflection of crustal isotherms should be reversed

from the deflection beneath sediment-draped topography because of the large variations in thermal resistance of the variable-thickness sediment cover. At any given depth below the sea floor, basement should be coolest, and thus downwelling should be located preferentially beneath basement ridges.

The heat-flow observations presented in Fig. 2 do not seem to support this conclusion, however, and an alternative hypothesis seems to be required. Our observations are most readily explained if there is sufficiently vigorous pore-fluid convection in the crust beneath the sediments to cause basement temperatures beneath the sediment seal to be generally uniform. Such vigorous small-scale convection within the upper crust could take place in addition to, and independent of, any larger scale forced circulation and associated recharge and discharge of fluids through the sediment section. This hypothesis can be examined with our data in a simple way. As discussed above, the sediment-basement contact, which is well imaged in the seismic profiles, probably defines a boundary between low- and high-permeability materials (a permeability contrast between the upper oceanic crust and the overlying sediments of from two to five orders of magnitude seems likely¹⁸⁻²³). Thus the temperatures estimated for this boundary should be representative of the temperatures in the upper part of the hydrothermal circulation system. Basement temperatures along the profile shown in Fig. 2, estimated by simple extrapolation of surface-gradient measurements down an estimated depth-dependent thermal-conductivity profile through the sediments, are remarkably uniform. They average 60 °C, and are locally scattered over a range of about ± 10 °C. We obtain a similar range of sediment-basement boundary temperatures for the entire area by plotting all heat-flow values obtained during the survey as a function of local sediment thickness (Fig. 3); we find strong inverse correlation between heat flow and sediment thickness seen along the profile shown in Fig. 2 throughout the study area.

Some of the scatter in the basement temperature estimates may be due to uncertainties in the estimates of the sediment thickness (the assumed thickness of the low-permeability layer) in this area of rough basement topography, although much of the scatter may represent real hydrothermal-fluid temperature variability. No systematic temperature variations can be seen that are correlated normally or inversely with the heat-flow variations (Fig. 3) or basement topography (Fig. 2), and thus at least at the limit of resolution of the basement temperature estimates, no hypothesis involving only topographically forced convection is supported. Unfortunately, the spacing of the measurements is too coarse for us to determine whether there is systematic basement temperature variability on a scale commensurate with that expected for unit-aspect-ratio convection in the upper few-hundred metres of the oceanic crust. Thus if the scatter in the temperature estimates is produced by small-scale convection beneath the sediments, the scale or pattern of the convection cannot be determined.

The 'vigour' of the hydrothermal circulation in the upper crust of this ridge flank can be described by the Rayleigh number (the degree of instability) that might characterize this convective system. Reasonable physical properties for sea water and basalt²⁶, a heat flow of 0.3 W m^{-2} , a layer thickness of 500 m, and a formation permeability of 10^{-13} m^2 yield a Rayleigh number of 360. This is well in excess of the critical value of 40 for convective instability. The predicted Nusselt number (the ratio of the total heat transfer in the convecting system to that which would be transferred by conduction only) for porous-medium convection at this Rayleigh number²⁷ is ~ 10 .

Admittedly, because of the very large uncertainty in the formation-scale permeability of the upper oceanic crust, these numbers are very approximate; they do demonstrate, however, that conditions permitting very efficient heat transport probably exist, and that the hypothesis is reasonable. The primary conclusion needs no qualification: considerable caution must be exercised in using heat-flow variations to infer the pattern of hydrothermal convec-

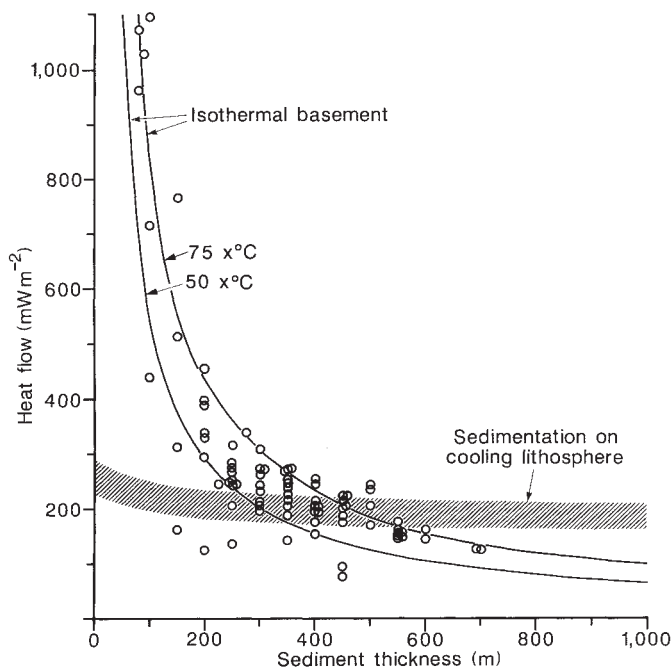


FIG. 3 Observed heat flow plotted against sediment thickness for all values in the survey area. Two hypothetical thermal conditions are represented by the shaded and solid lines: (1) heat flow is roughly uniform between 200 and 300 mW m^{-2} in the area (corresponding to the conductive cooling of 2.8 to 5-Myr-old sea floor), but locally depressed by the thermal effects of sedimentation (shaded area); (2) heat flow varies inversely with sediment thickness because the basement-sediment interface is maintained at a roughly constant temperature by vigorous hydrothermal circulation in the upper crust (75 °C and 50 °C for the upper and lower lines, respectively). The observations support the hydrothermal circulation hypothesis.

tion, particularly if there are significant variations in the sediment thickness. All of the significant heat-flow variations in this ridge-flank setting can be explained by sediment thickness variations; little 'signal' remains that can be used to constrain the pattern of crustal hydrothermal convection.

Further constraints provided by sediment pore-fluid geochemical gradients have been used to characterize the rate and pattern of fluid flux through sediments²⁴, and numerical simulations have been used to provide estimates of flow rates and other characteristics of circulation at depth²⁴. Similar geochemical measurements and numerical studies, along with pore-fluid pressure measurements and further and more closely spaced heat-flow measurements over both rough and smooth basement topography are planned for this area of the Juan de Fuca Ridge flank in 1990. □

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Electrical conductivity of the Earth's lower mantle

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ELECTRICAL conductivity is an important physical property of the Earth's mantle because it controls the transmission of geomagnetic signals from the core to the surface. The lower mantle, from a depth of 670 km down to the core–mantle boundary (2,990 km), is probably composed of (Mg,Fe)SiO₃ perovskite and magnesio-wüstite, (Mg,Fe)O. Analysis of the transient and secular variations of the geomagnetic field yields values of the lower-mantle conductivity of the order of 1 S m⁻¹ at a depth of 1,000 km, increasing to ~100 S m⁻¹ at the core–mantle boundary^{1,2}. Information about the physical mechanism of the conductivity and its dependence on temperature and pressure would help to constrain the temperature profile in the Earth. In the only study published so far, Li and Jeanloz^{3,4} reported values of the conductivity of the silicate perovskite and of a mixture of perovskite and magnesio-wüstite lower than 10⁻³ S m⁻¹ and concluded that the lower mantle is an insulator, thus casting doubt on the geomagnetic results. We report here the results of measurements of the d.c. electrical

conductivity of a mixture of perovskite and magnesio-wüstite resulting from disproportionation of olivine, and of pure perovskite. The measurements were made in an externally heated diamond-anvil cell at pressures of ~40 GPa and temperatures from 25 °C to ~400 °C. Conductivity increases with increasing iron content, increasing temperature and increasing pressure. The activation energy (0.35 eV for 11% Fe) decreases with increasing iron content. The results are compatible with an electron-hopping conduction mechanism. Extrapolation to the temperature appropriate for a depth of 1,100 km, which corresponds to the pressure of our experiments, yields a conductivity of the lower mantle of the order of 1 S m⁻¹; extrapolation to the temperature and pressure of the core–mantle boundary yields values between 50 and 100 S m⁻¹, in agreement with geomagnetic determinations.

The starting materials that we used were olivine and enstatite crystals from San Carlos xenoliths, with an iron content of 11 and 16 atom% and 8 and 11 atom% respectively (determined by electron-microprobe analysis). We ground the crystals under alcohol in an agate mortar and dried them in a microwave oven. This powder was loaded into a diamond-anvil cell, without gasket, between two parallel tungsten ribbons that were separated by 0.08 mm and used as leads (Fig. 1). We fitted a wire-

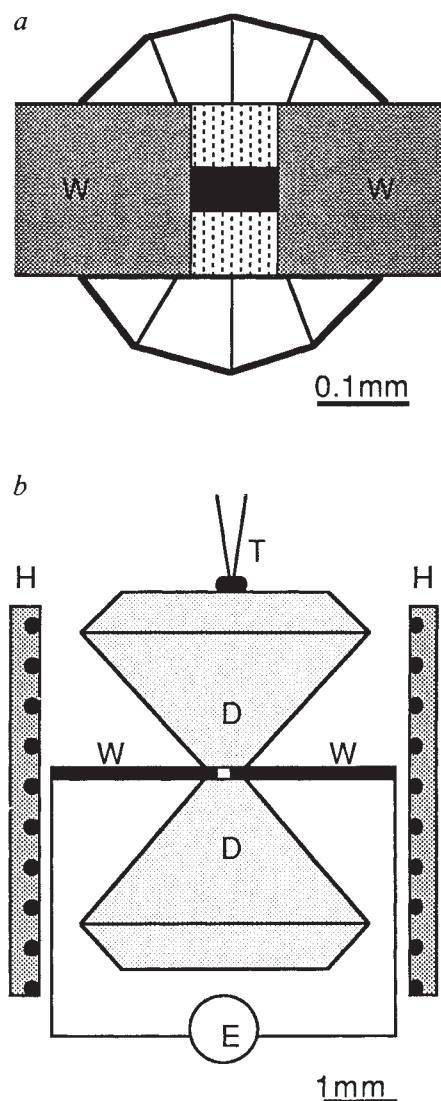


FIG. 1 a, Top view of the assembly: The sample (black) is prepared by transforming the olivine or enstatite (stippled) between the tungsten leads (W). b, Schematic view of the apparatus: D, diamonds; W, tungsten leads; T, thermocouple; H, heater; E, electrometer.

wound resistive heater around the diamonds and used a thermocouple in contact with the back of one diamond to measure temperature to $\pm 10^\circ\text{C}$. With the cell at room temperature, we increased the pressure to values in the range 37–52 GPa, within the stability field of perovskite; to avoid contamination, we measured the peak pressure, reasonably uniform over the sample in the central zone of the cell, using a force gauge calibrated with the ruby scale, it is therefore not known with a great accuracy (± 2 GPa) but the reproducibility is good. We measured the electrical resistance under a constant voltage of 2 V with a Keithley 617 electrometer. The input impedance of the electrometer was 200 T Ω and if the electrometer is used as an ohmmeter, resistances up to 200 G Ω can be measured. By using the internal voltage source, however, we could measure resistances up to 10,000 T Ω , with a relative resolution of 5×10^{-5} . The nominal accuracy is given as 1.5%. We measured the resistance of the apparatus and untransformed material at room temperature before each experiment and found it to be $\sim 10^{12} \Omega$. We also measured it with increasing temperature and found it to be $\sim 10^{11} \Omega$ at 220°C , four orders of magnitude higher than the resistance of the transformed sample.

We prepared the sample by translating the cell under the laser

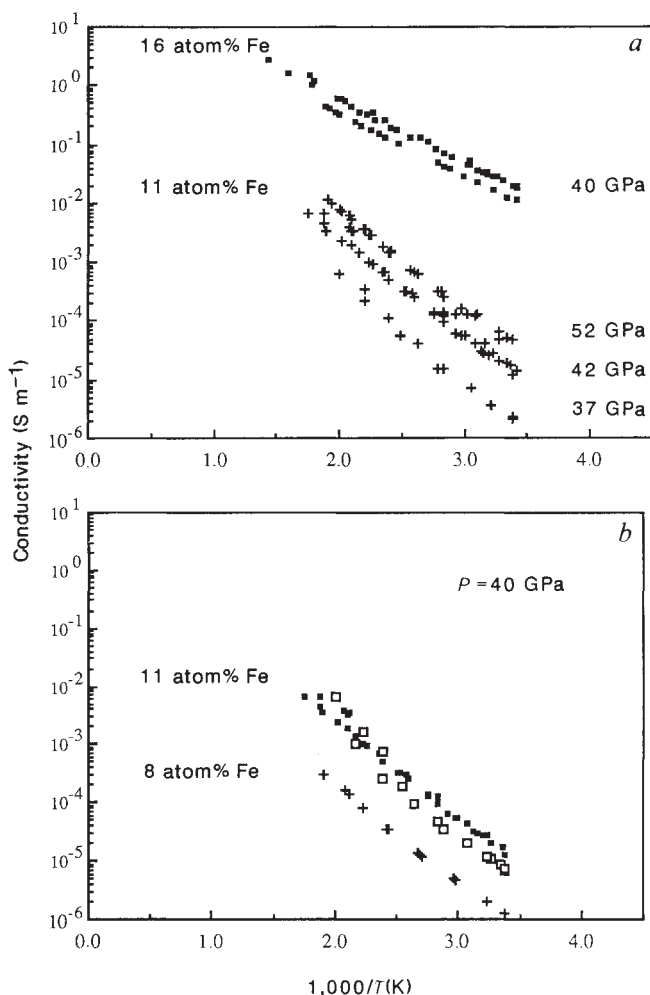


FIG. 2 *a*, Arrhenius plot of the electrical conductivity of the perovskite and magnesiowüstite mixtures obtained from the disproportionation of San Carlos olivines with 11 and 16 atom% Fe. Synthesis pressure from 37 to 52 GPa. Temperatures from 25°C to 298°C (for 11 atom% Fe) and 426°C (for 16 atom% Fe). *b*, Arrhenius plot of the electrical conductivity of perovskite synthesized at 40 GPa from San Carlos enstatite with 8 atom% Fe (crosses) and 11 atom% Fe (empty squares). The Arrhenius plot for a perovskite and magnesiowüstite mixture, synthesized at about 42 GPa from San Carlos olivine with 11 atom% Fe, is given for comparison (full squares).

beam (15-W continuous-wave Nd YAG) so that a $80 \times 30 \times 10\text{-}\mu\text{m}$ strip of perovskite and magnesiowüstite (or perovskite alone) was formed between the leads. The room-temperature and high-temperature resistance of this strip was orders of magnitude smaller than that of the untransformed material. To make sure that the resistance measured was indeed that of the strip of transformed material, a second identical strip was made and we checked that the total resistance was half that measured for a single strip. Finally, we checked that by completely decompressing the sample and running the laser transversely to the strips, thus transforming the material back to olivine, the circuit was broken and the resistance came back to its high initial value.

For every starting material synthesized at a given pressure and kept in the cell at the synthesis pressure, several runs were made under identical conditions. For each run, we measured the resistance on increasing the temperature to 300 or 400°C and down to room temperature again; one to three cycles were usually performed with excellent reproducibility. We estimate the accuracy on the resistance measurement and the sample dimensions to be $\sim 10\%$, and total accuracy on the value of the conductivity to be $> 50\%$.

The experimental results are shown in Figs 2 and 3. We find that: (1) Electrical conductivity of perovskite is not significantly different from that of the mixture of perovskite and magnesiowüstite having the same iron content (11 atom% Fe). (2) Conductivity increases with increasing iron content. At room temperature and 40 GPa, it is $\sim 2 \times 10^{-5} \text{ S m}^{-1}$ for 11 atom% Fe and $2 \times 10^{-2} \text{ S m}^{-1}$ for 16 atom% Fe. (3) Conductivity increases with temperature according to an Arrhenius law, the activation energy decreases with increasing iron content ($E_a = 0.35 \text{ eV}$ for 11 atom% Fe, $E_a = 0.21 \text{ eV}$ for 16 atom% Fe). (4) Conductivity increases with pressure (synthesis pressure, kept constant during the measurement), it also increases, although less rapidly, with pressure applied to the sample. (5) The activation energy for a given iron content does not significantly depend on synthesis pressure in the narrow range of pressures investigated.

The results so far are compatible with a charge-transfer conduction mechanism by hopping of holes between Fe^{3+} and Fe^{2+} ions, already documented in the case of transition-metal olivines⁵ and magnesiowüstites^{6–8}. Activation energies are similar to those found for hopping conduction in magnesiowüstites^{7,8} and the fact that conductivity increases with pressure is compatible with this mechanism. Hopping conduction, of course, depends on the $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio, which in turn depends on oxygen partial pressure. Our starting material came from the same locality and in one case (11 atom% Fe) from the same xenolith; it was loaded in the cell in air. It is therefore probably not unreasonable to assume that the (as yet unknown) oxygen partial pressure was the same in all our experiments and resulted from the equilibration of similar material in the cell at high temperature. No perceptible oxidation took place during heating—the conductivity at room temperature was unchanged after

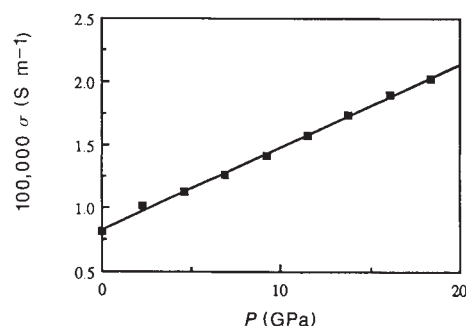


FIG. 3 Effect of pressure on the room-temperature electrical conductivity of the perovskite and magnesiowüstite mixture synthesized at 40 GPa from San Carlos olivine with 11 atom% Fe, decompressed to ambient pressure and recompressed up to 18 GPa. The rate of increase of the conductivity is $\sim 7 \times 10^{-7} \text{ S m}^{-1} \text{ GPa}^{-1}$.

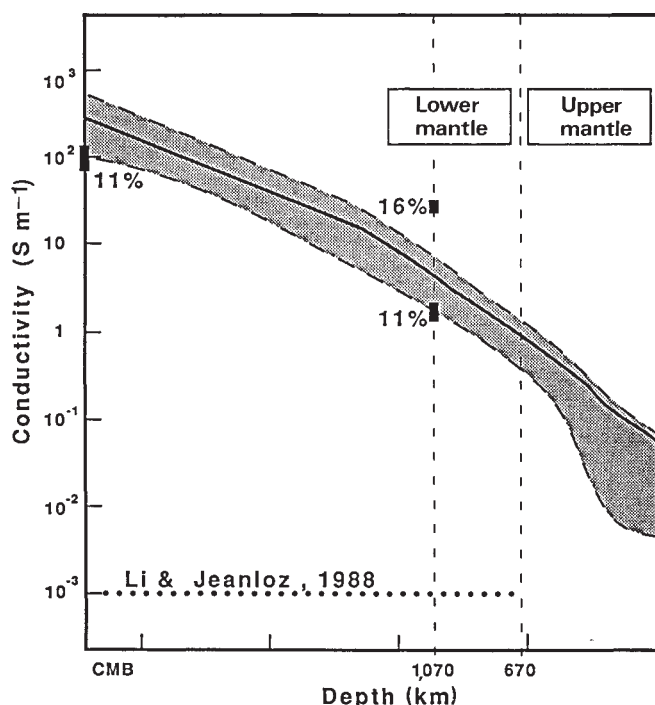


FIG. 4 Electrical conductivity of perovskite and magnesiowüstite assemblages (11 and 16 atom% Fe), extrapolated to the lower-mantle conditions at depths of 1,070 and 2,990 (core-mantle boundary, CMB) km. The stippled band corresponds to the conductivity profile determined from geomagnetic data¹. The dotted line is the upper limit to the conductivity of the lower mantle given in ref. 4.

several heating and cooling cycles. Further investigation of the effect of oxygen partial pressure is under way. The temperature gradient at the edges of the laser beam is extremely high as temperature increases by almost 3,000 K over a few micrometres. Under these conditions iron might migrate away from the heated area⁹ and cause drastic changes in electrical conductivity; however, in our experiments the laser is scanned across the sample in a few seconds, whereas Heinz and Jeanloz heated a spot for 45 min. We nevertheless checked that no change in composition had occurred during synthesis by examining a sample with analytical transmission electron microscopy, using the technique described in ref. 10: a transverse profile across the sample showed that, within the experimental error of 0.5%, there was no iron enrichment toward the edges. We could also check that the sample was indeed perovskite and that no other phase was present in the high-temperature zone.

A pressure of 42 GPa corresponds to a depth of 1,070 km in the Earth¹¹, where temperature is probably in the range 2,200–2,500 K (refs 12, 13). If we make the conservative assumption that no mechanism with a higher activation energy takes over at >400 °C and extrapolate our data at constant pressure to 2,200–2,500 K, we find values for the lower-mantle conductivity in the range 4–40 S m⁻¹, depending on the iron content. If we extrapolate our results to the temperature and pressure of the core-mantle boundary (3,000 K, 135 GPa), the conductivity is ~70 S m⁻¹. The uncertainty is obviously much greater here, because the extrapolation in temperature is made over a greater range and because the extrapolation in pressure is even less well constrained. We feel, however, that extrapolation with a constant activation energy probably leads to a lower bound on conductivity, because it is hard to think of any mechanism whereby the conductivity might substantially decrease with increasing temperature. Indeed, it is usually the case that mechanisms with higher activation energy are effective at higher temperatures (such as in ionic conduction) and the slight upward curvature apparent in our Arrhenius plots might perhaps be interpreted

as indicating a high-temperature regime. These conclusions hold whether the lower mantle is pyrolytic¹⁴, with ~80 vol% perovskite as would result from disproportionation of olivine, or chondritic¹⁵, with up to 100% perovskite, and whether it is enriched in iron with respect to the upper mantle¹⁶ or not.

These results are in contradiction with those of Li and Jeanloz, who gave 10⁻² S m⁻¹ (ref. 3) and more recently 10⁻³ S m⁻¹ (ref. 4) as an upper limit to the electrical conductivity of (Mg_{0.9}Fe_{0.1})SiO₃ perovskite at lower mantle conditions. They are in good agreement, however, with the results derived from the analysis of the long-period time variations of the geomagnetic field^{1,2} (Fig. 4). □

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Rapid dwarfing of red deer on Jersey in the Last Interglacial

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THE dwarfing of large mammals on islands occurred repeatedly in the Pleistocene. Elephants, deer, hippopotami and other species became dwarfed on islands in Indonesia, the Mediterranean, the east Pacific and elsewhere^{1,2}. In most cases, the full-sized ancestral form can be recognized among the adjacent mainland fauna, but evolutionary rates cannot be estimated because the entry of the ancestor onto the island, and appearance of the dwarf form, are poorly dated. Here I give the first example in which the island dwarf is well dated, the full-sized ancestor is found in demonstrably older deposits on the island, and a good estimate can be made for the duration of the isolation leading to dwarfing. In the Last Interglacial, red deer on Jersey, Channel Islands, became reduced to one sixth of their body weight in less than six thousand years.

Jersey, an island of about 130 km², lies some 25 km from the coast of France (Fig. 1). Belle Hougue cave, on the north of the island, contains a raised beach deposit at 8 m above Ordnance Datum (+8 m OD) which yielded remains previously identified on antler features as a dwarfed form of red deer, *Cervus elaphus* L.³. This identification is confirmed by study of detailed morphological characters on the 36 fossil antlers, teeth and postcranial elements (representing a minimum of five individuals), in comparison with material of all known cervid species of the European Middle and Upper Pleistocene⁴. A uranium-series date of 121⁺¹⁴₋₁₂ kyr on travertine cementing the raised beach, together with amino-acid racemization studies of molluscan shells, imply a Last Interglacial (Eemian) age for the deposit^{5,6}.

Size estimates of the deer are based on fully grown adult specimens (fused limb bones and permanent dentition). In comparison with mainland Eemian red deer, the Belle Hougue fossils

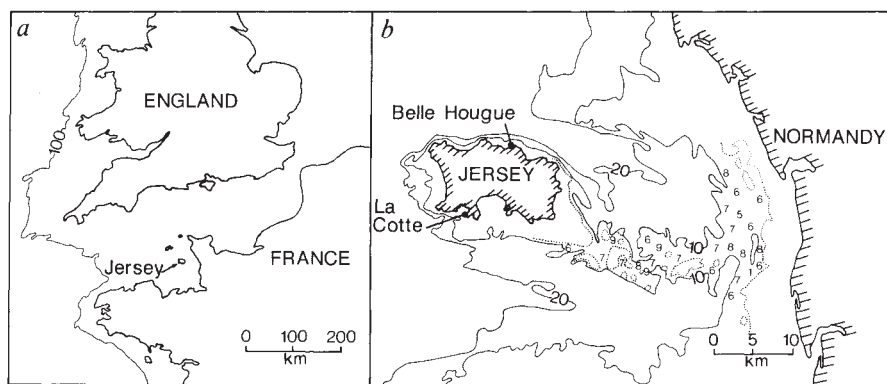


FIG. 1 a, Location map of Jersey, showing 100 m submarine contour. b, Twenty- and ten-metre contours around Jersey, from ref. 12. Depths are reduced to Chart Datum, which is approximately the level of the lowest astronomical tide. The 5 m contour (dotted), and point soundings between 5 and 10 m, are shown for the submerged isthmus between Jersey and the mainland.

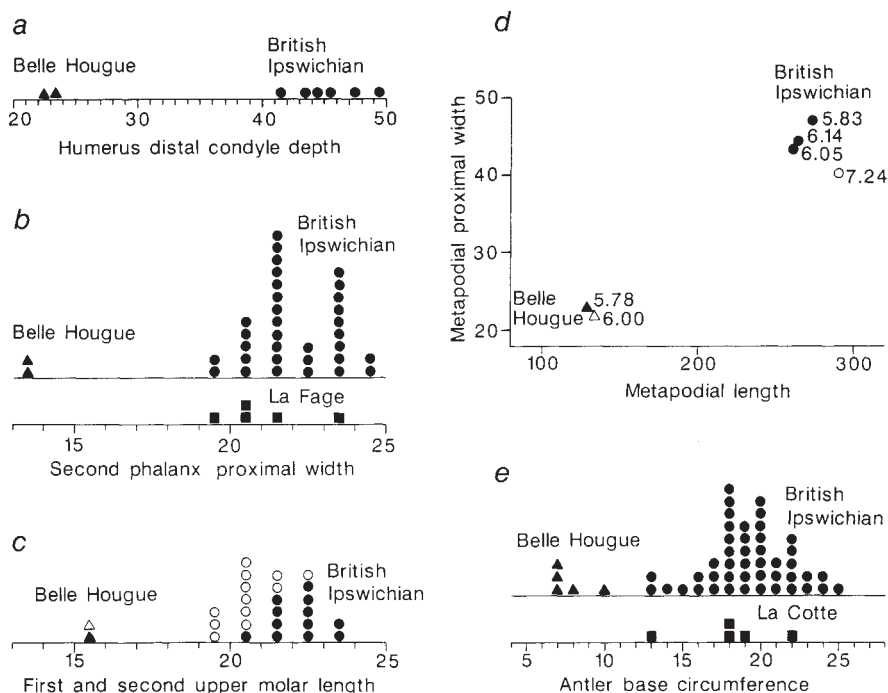
show ~56% reduction in limb bone diameters (Table 1; Fig. 2a, b and d). This measure, which is approximately geometrically related to body weight among deer⁷, implies a weight reduction of about six times, from ~200 kg in males of the ancestral population (A.M.L., manuscript in preparation) to ~36 kg in the Jersey dwarf. Teeth were less reduced in size than the postcranial skeleton (Table 1; Fig. 2c), as found in many dwarf forms⁸, and antlers were simplified in ways similar to those seen in other small-bodied populations of red deer^{3,9}. Relative shortening of the lower limb bones (metapodials), common in island dwarfs¹, may have occurred to a limited extent (Fig. 2d), but the sample is too small for certainty.

At La Cotte, on the southern side of Jersey, a sequence of deposits dates from the Saalian cold stage, when low sea level meant that Jersey was part of the broad plain of northwestern France. Red deer have been recovered from a number of Saalian horizons at La Cotte, the lowest dated by thermoluminescence to 238 ± 35 kyr, the highest succeeded by an Eemian raised beach at +9 m OD^{10,11}. Only antler remains are complete enough for standard measurements, but the size of these and other fragmentary fossils (28 specimens in total)¹⁰ clearly indicates a red deer of 'normal' size, similar to Saalian and Eemian remains from mainland Britain and France (Fig. 2e). Thus they illustrate the large-bodied population that existed on Jersey before the Eemian isolation and dwarfing, and which on the mainland continued almost unchanged throughout the Eemian.

Today, a sea-level drop of only 8 m would be sufficient to connect Jersey to mainland Normandy by a narrow isthmus at the lowest tide¹² (Fig. 1). This would imply that Jersey was fully isolated in the Eemian only when mean sea level was above -8 m OD, provided the present situation is not the result of post-Eemian uplift. Such uplift appears to have been minimal, judging from the +8 m OD height of the Eemian raised beaches on Jersey, which are mirrored by Eemian maximum deposits at similar height in northern France and southern England^{6,13-15}, and differ only slightly from the +6 m OD Eemian maximum global-eustatic sea level evidenced by oxygen isotope and coral terrace data^{16,17}. If real, the small difference between these figures could be due to the large tidal range of the area, and/or to 2 m of uplift at most. Allowing for the latter, we can take a mean sea level of higher than -10 m OD as providing full isolation of Jersey in the Last Interglacial.

Data from deep-sea cores and from varved lake deposits independently indicate that the Eemian (oxygen isotope stage 5e) lasted ~11 kyr^{18,19}, from 126 to 115 kyr (ref. 18). It can be calculated from oxygen isotope ratios that mean sea level was above -10 m OD (sufficient to isolate Jersey) for 9-10 kyr during this period (Fig. 3a). A similar figure can be deduced from terrestrial sequences. Evidence from the Netherlands shows that sea level rose rapidly during Eemian pollen zones 1-4, reached its maximum stand during zone 5, and fell during zone 6 (ref. 15), a pattern corroborated by data from southern Britain and

FIG. 2 Comparison of red deer from Belle Hougue and La Cotte with mainland Saalian and Ipswichian (Eemian) samples, the latter pooled from sites listed in Table 1. a, Humerus distal condyle depth: ▲, Belle Hougue; ●, British Ipswichian. b, Second phalanx proximal width: ▲, Belle Hougue; ●, British Ipswichian; ■, French Saalian (La Fage) from ref. 24. c, First and second upper molar length: △, Belle Hougue M¹; ▲, Belle Hougue M²; ○, British Ipswichian M¹; ●, British Ipswichian M². d, Metapodial length and proximal width: ▲, Belle Hougue metacarpal; △, Belle Hougue metatarsal; ●, British Ipswichian metacarpals; ○, British Ipswichian metatarsal. Number by each point is the ratio of length/proximal width. e, Antler base circumference: ▲, Belle Hougue; ■, La Cotte; ●, British Ipswichian. Antler measurement was taken below the rose, around the pedicle scar. Mainland sample spans young (smaller) and mature antlers; La Cotte sample comprises one young and four mature antlers; Belle Hougue antlers are all mature, as shown by their branch development and short pedicles³.



northern France¹³⁻¹⁵. Taking the zone 5 stand to correspond to the +6 m eustatic maximum, mean sea level was above -10 m OD from approximately the zone 3/4 boundary to the end of zone 6 (Fig. 3b). Varved Eemian lake sediments in Germany indicate that this period (pollen zones 4, 5 and 6) probably lasted 8-9 kyr (Fig. 3b)¹⁹.

However, the dwarfing probably occurred in less than the total time of isolation. The Belle Hougue beach is at +8 m OD, indicating that it was deposited close to the time of maximum sea level, during pollen zone 5. This narrows the time available for dwarfing to the interval from the zone 3/4 boundary (when Jersey became isolated), to an unknown point in zone 5 (when the beach formed). As zones 4 and 5 lasted ~1,800 and ~4,000 yr respectively¹⁹, this gives an available time span of up to ~5,800 yr (Fig. 3b). Even if mean sea level had been a few metres below the beach, this time span would be increased by only a few hundred years, given the rapid fall in sea level after pollen zone 5 (Fig. 3b).

Although red deer show marked ecophenotypic size changes²¹, a sixfold weight reduction was probably largely the result of selection, a response to resource limitation and freedom from predation^{1,22}. Deer are good swimmers, so the dwarfing of the Jersey population implies considerable geographic isolation to prevent hybridization with mainland animals. It has been suggested¹ that the evolution of island forms depends on the possibility of an occasional crossing from the mainland, allowing initial colonization. In the case of the Jersey deer, however, it is just as likely that the dwarf form evolved from normal-sized deer already present on the island (the La Cotte population, or later Saalian immigrants), which became isolated by the high sea level of the Eemian.

The origin of dwarfed red deer on Jersey is an example of rapid evolution in an allopatric isolate. The time taken, 6 kyr or less, is geologically extremely short; mainland European red deer had existed with only minor changes for the previous 400 kyr^{4,23}. On the other hand, we cannot know whether speciation (in the sense of reproductive isolation) occurred, and the dwarf population seems not to have survived the re-establish-

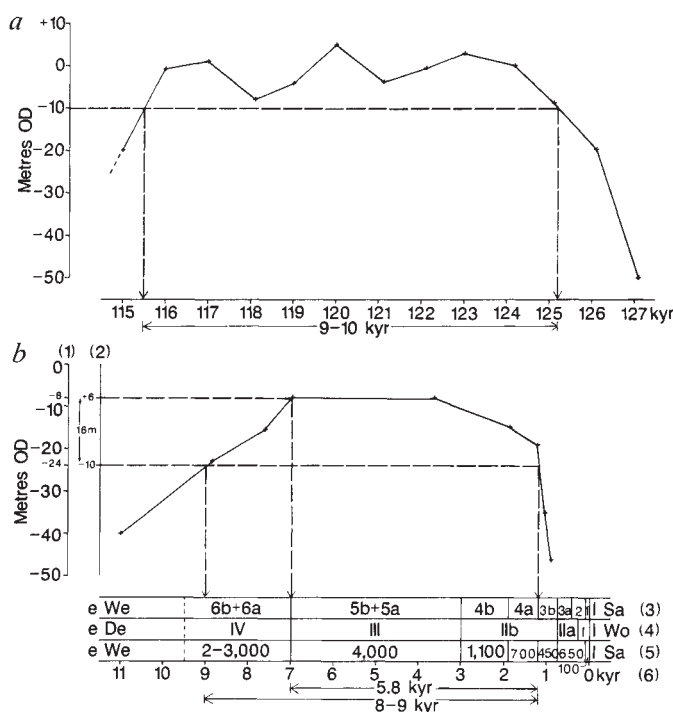


FIG. 3 Eemian sea-level changes and the isolation of Jersey. *a*, Curve based on oxygen isotope ratios and interpolated dates, from ref. 17. *b*, Curve based on marine transgressions in the Netherlands in relation to pollen zonation. Curve *b* is replotted from ref. 20 onto absolute timescale from ref. 19. Axes represent: (1) Present height of deposits in the Netherlands, the entire sequence having been downwarped since the Eemian²⁰; (2) height of the deposits in the Eemian, assuming the maximum stand to have been at +6 m OD^{16,17}; (3) Dutch pollen zones²⁰; (4) equivalent British pollen zones²⁵; (5) duration of pollen zones¹⁹; (6) timescale in kyr since the beginning of the interglacial, based on (5); (7) range due to uncertainty of correlation²⁰ between pollen zonation in refs 13 and 19. Abbreviations: e We, early Weichselian; e De, early Devensian; l Sa, late Saalian; l Wo, late Wolstonian.

TABLE 1 Measurements of dwarf red deer from Belle Hougue Cave, Jersey, in comparison with contemporary mainland British Ipswichian (Eemian) samples

Element	Measurement	Belle Hougue specimen	Mean	British Ipswichian s.d.	n	Belle Hougue as % of British Ipswichian mean
Teeth						
P ⁴	Length	10.0	12.20	—	1	82.0
M ¹	Length	15.7	20.49	0.884	12	76.6
M ²	Length	15.1	22.09	1.026	12	68.4
M ₁	Length	15.0	18.85	0.889	4	79.6
M ₂	Length	17.1	22.50	1.584	5	76.0
						Mean (n=5) 76.5%
Limb bones						
Scapula	glenoid w.	25.8	—	—	0	—
Scapula	glenoid d.	25.0	—	—	0	—
Humerus	dist. condyle d.	23.4	45.18	3.008	6	51.8
Humerus	dist. condyle d.	22.1	45.18	3.008	6	48.9
Radius	prox. w.	35.5	62.95	3.465	4	56.4
Metacarpal	prox. w.	22.5	43.17	2.104	6	52.1
Femur	prox. head w.	23.0	38.00	—	1	60.5
Tibia	dist. w.	29.0	53.10	3.513	5	54.6
Metatarsal	prox. w.	22.0	39.83	1.097	3	55.2
Metatarsal	prox. w.	22.5	39.83	1.097	3	56.5
Phalanx II	prox. w.	13.3	21.84	1.322	33	60.9
Phalanx II	prox. w.	13.4	21.84	1.322	33	61.6
						Mean (n=10) 55.9%

Measurements are given in mm. Each row represents a single Belle Hougue specimen. Abbreviations: prox, proximal; dist, distal; w, width; d, depth. Mainland sample pooled from Histon Road and Barrington, Cambs.; Easton Torrs, Tornewton and Joint Mitnor Caves, Devon; Bacon Hole, Gower; Trafalgar Square, London; Swanton Morley, Norfolk; Milton Hill, Somerset; Kirkdale and Victoria Caves, Yorks; for site details see ref. 26. Belle Hougue measurements are expressed as per cent of mean for the corresponding element in the Ipswichian sample, and then pooled to provide an estimate of the average reduction in tooth length and limb bone diameter (see ref. 27 for a discussion of the technique). Specimens from mainland British Wolstonian (Saalian) localities, and from the 'Ilford' group of sites²⁶ are of similar size to the Ipswichian material listed here (A.M.L., manuscript in preparation).

ment of a land link at the end of the Eemian. It succumbed to mainland predators¹, or to competition from other herbivores, or conceivably to hybridization with mainland red deer. □

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Genetic correlations between morphology and antipredator behaviour in natural populations of the garter snake *Thamnophis ordinoides*

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THE genetic coupling of morphology and behaviour means that the evolution of the two types of traits will not be independent: changes in behaviour will result in changes in morphology and vice versa^{1,2,3}. This might explain nonadaptive differences in morphology through indirect selection on correlated characters of other categories⁴. Genetic correlations between morphology and behaviour are also the basis for some models of sympatric speciation⁵ and of the stability of polymorphisms⁶. Morphology and behaviour are often correlated in nature^{7–11} and a genetic basis for such couplings has been demonstrated^{12,13}. I present here evidence that colour pattern and antipredator behaviour are genetically coupled in natural populations of the garter snake *Thamnophis ordinoides*. Similar phenotypic correlations between pattern and behaviour exist among species of North American snakes¹⁴, indicating that selection for particular combinations of traits may help to maintain genetic covariances and colour polymorphism in *Thamnophis ordinoides*.

Interspecific studies can provide a basis for predicting patterns of genetic correlations within populations. Dorsal pigmentation

patterns of different species of snakes are correlated with specific ecological and behavioural tendencies. Longitudinally striped snakes tend to be more active, wide-foraging and flee when threatened, whereas species with broken colour patterns (blotches or crossbands) are more secretive and rely on crypsis or aggression as their primary defence¹⁴. These relationships probably reflect survival advantages of particular combinations of traits. It is more difficult for the vertebrate eye to detect motion or judge the speed of a moving stripe than a moving heterogeneous pattern¹⁵, so that flight may be a more effective antipredator behaviour for striped snakes than for blotched ones.

The Northwest garter snake, *Thamnophis ordinoides*, exhibits variation in colour pattern within populations similar to that observed across snake species. To determine whether this morphological variation is genetically coupled with behavioural variation, colour patterns and four types of antipredator behaviour¹⁶ were scored for 474 neonates from 77 litters from 3 natural populations of *Thamnophis ordinoides* in coastal Oregon (Table 1). The four behaviours scored (sprint speed, endurance, antipredator display and the number of reversals during flight) were not mutually exclusive, but rather were each present in varying degrees in each individual.

Neither repeatabilities nor heritabilities differed significantly among populations. Pooled estimates and population estimates are given in Table 1. Estimates of repeatabilities indicated significant ($P < 0.05$) variation among individuals for each of the antipredator behaviours scored. The broad-sense heritabilities of all behaviours and the stripe index were significantly different from zero, indicating the existence of significant additive genetic variance for each of these traits (broad-sense heritability estimates also include a fraction of dominance variance²).

Colour pattern was genetically correlated with the number of reversals during flight but not with any other behaviour

TABLE 1 Repeatabilities (R) and heritabilities (h^2) $\pm$ standard error for all traits

		Tenmile (200, 29)	Alsea (122, 19)	McGribble (152, 29)	All (474, 77)
Stripe	R	—	—	—	—
	h^2	0.707 $\pm$ 0.162	0.511 $\pm$ 0.207	1.080 $\pm$ 0.170	0.781 $\pm$ 0.008
Distance	R	0.725 $\pm$ 0.024	0.823 $\pm$ 0.024	0.767 $\pm$ 0.044	0.773 $\pm$ 0.016
	h^2	0.798 $\pm$ 0.164	0.575 $\pm$ 0.212	0.387 $\pm$ 0.169	0.593 $\pm$ 0.103
Reversal	R	0.465 $\pm$ 0.033	0.509 $\pm$ 0.052	0.348 $\pm$ 0.082	0.456 $\pm$ 0.004
	h^2	0.245 $\pm$ 0.122	0.225 $\pm$ 0.110	0.783 $\pm$ 0.184	0.366 $\pm$ 0.007
Display	R	0.536 $\pm$ 0.031	0.546 $\pm$ 0.049	0.509 $\pm$ 0.074	0.534 $\pm$ 0.004
	h^2	0.161 $\pm$ 0.112	0.630 $\pm$ 0.216	0.513 $\pm$ 0.178	0.377 $\pm$ 0.007
Speed	R	0.528 $\pm$ 0.032	0.840 $\pm$ 0.021	0.766 $\pm$ 0.041	0.728 $\pm$ 0.003
	h^2	0.528 $\pm$ 0.032	0.419 $\pm$ 0.196	1.173 $\pm$ 0.163	0.608 $\pm$ 0.005

Estimates of repeatabilities are from intraclass correlations^{2,22} of four repeated measures for sprint speed and two repeated measures for other behaviours. The average of all three populations (weighted by the reciprocal of each population's sampling variance) is given under 'All'. The number of individuals and families, respectively, for each population is shown in parentheses. Specific components of colour pattern were scored and then combined into an index indicating 'stripedness'. Pattern components are mendelian characters (S. J. Arnold, unpublished data) and are combined to create a quantitative character more appropriate for quantitative genetic analyses. The index used is $\{(DS \times DC) + (LS \times LC) + 1\} / SPOT = STRIPE$, where DS and LS are the completeness of dorsal and lateral stripe on a 0–4 scale (0=absent, 4=present the entire length of the body), DC and LC are the contrast of dorsal and lateral stripe on a 0–3 scale and SPOT is the presence or absence of spots (1=no spots, 2=1 row and 3=2 rows). Neonatal locomotor performance (sprint speed and endurance), defensive display²³ and the number of reversals in direction during flight¹⁶ were scored repeatedly at the same age (3–6 days old) for each individual¹⁶. Regression techniques were used to statistically remove the maternal effects associated with condition of the female for all behaviour scores²⁴. Residual behaviour scores from a regression on female mass and snout-vent length were used to calculate heritabilities and genetic covariances (Table 2). Additive genetic parameters were estimated by analyses of variance of covariance within and among presumed full-sib litters and therefore may include contributions from dominance deviations and the prenatal common environment².

TABLE 2 Genetic correlations between stripe and behaviour scores for each population

Tenmile (29)	Distance	Reversal	Display	Speed
Stripe	-0.191	-0.751	0.150	0.280
	-0.173	-0.427**	0.106	0.219
Alsea (19)				
Stripe	0.384	-1.200	-0.180	0.635
	0.196	-0.499**	-0.060	0.291
McGribble (29)				
Stripe	0.491	-0.184	0.495	0.293
	0.187	-0.012	0.543*	0.303

The top row indicates covariance component estimate of the correlation based on full-sib data^{2,22}. Litter mean correlations are presented on the second row for purposes of significance testing^{4,25}. The number of litters for each population is shown in parentheses. Asterisks indicate the significance level of the litter mean correlation (** = $P < 0.01$; * = $P < 0.05$). See Table 1 for methods.

examined (Table 2). In two populations, significant negative genetic correlations were detected between the stripe index and the number of reversals during flight. In the third population, a significant correlation was observed between the stripe index and antipredator display, but this correlation was due almost entirely to a single family and it was disregarded.

Sudden terminations of flight, such as the reversals scored here, can cause a predator to lose track of its quarry, allowing the prey a second chance to use crypsis as a defence^{16,17}. The correlations observed in the Tenmile and Alsea populations indicate that striped snakes flee directly whereas unstriped and spotted snakes exhibit a greater tendency for cryptic behaviour. This resembles the interspecific correlations between colour pattern and antipredator behaviour observed among all North American snakes¹⁴.

Genetic covariances result from pleiotropy (whereby genes affect multiple traits) or linkage disequilibrium (nonrandom association of alleles at different loci) or both. The data presented here cannot be used to distinguish between these causes, but the genetic covariances probably result from linkage disequilibrium rather than pleiotropy, because the coupling is between morphological and behavioural traits. In either case, theory indicates that genetic covariances can be maintained by selection for particular combinations of traits¹⁸. The concordance between the correlations detected in this study and those observed across snake species indicates that selection favouring particular combinations of colour pattern and behaviour may be at least partially responsible for the maintenance of the genetic covariances reported here. Other phenomena that may contribute to the maintenance of genetic correlations include assortative mating, secondary contact of divergent populations, small effective population size and population subdivision¹⁹.

Selection favouring combinations of traits could also help to explain the extensive colour pattern polymorphism within populations of *Thamnophis ordinoides*. If the fitness of an individual with a particular colour pattern depends on its antipredator behaviour, then it may be that individuals with different combinations of colour pattern and antipredator behaviour have comparable fitnesses. A similar argument has been used to explain polymorphism in heterogeneous environments where different genotypes choose to occupy the microhabitat in which they are most fit. Under these conditions genetic polymorphisms within populations can be maintained with small genetic loads⁶.

The post-selection distribution of all characters will be a result not only of direct selection on those characters, but also of selection on genetically correlated characters. In this way, genetic covariances can constrain the rate²⁰ and even determine the direction²¹ of short-term evolutionary change. Thus, genetic correlations between morphology and behaviour imply a high degree of genetic integration of the entire phenotype, indicating that phenotypic evolution is more complex than even multivariate analyses of a single category of characters would indicate. □

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Independent hemispheric attentional systems mediate visual search in split-brain patients

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THE primate visual system is adept at identifying objects embedded within complex displays that contain a variety of potentially distracting elements. Theories of visual perception postulate that this ability depends on spatial selective attention, a mechanism analogous to a spotlight or zoom lens, which concentrates high-level processing resources on restricted portions of the visual field^{1,2}. Previous studies in which attention was pre-cued to specific locations in the visual field have shown that the spotlight has a single, unified focus^{2,3}, even in the disconnected hemispheres of patients who have undergone surgical transection of the corpus callosum^{4,5}. Here we demonstrate that an independent focus of attention is deployed by each of the surgically separated hemispheres in a visual search task, such that bilateral stimulus arrays can be scanned at a faster rate by 'split-brain' subjects than by normal control subjects. The attentional system used for visual search therefore seems to be functionally and anatomically distinct from the system that mediates voluntary orienting of attention.

In visual search tasks of the type studied here, an array of items is presented and the subject must decide whether or not a specified target item is present in the array. When the target and distractor items share the same basic features but differ in combinations or conjunctions of these features, attention must be focused on each item in turn (serial search) until the target is found or the array is exhausted^{1,6}. Reaction time therefore increases linearly with the number of items in the array (the set size), and the slope of the function relating reaction time to set size indicates the amount of time required to scan each item (the search rate).

Several studies in normal subjects have indicated that the

TABLE 1 Mean per cent correct for the control subjects and split-brain patients

		Set size		
		2	4	8
Control	Unilateral	98.5 (1.6)	97.6 (2.4)	94.9 (2.9)
	Bilateral	99.6 (0.6)	99.4 (0.7)	97.9 (2.4)
Split	Unilateral	99.4 (0.8)	96.1 (5.5)	92.2 (1.6)
	Bilateral	98.3 (1.1)	98.4 (0.8)	97.2 (4.0)

Standard deviations are shown in parentheses.

attentional spotlight is unitary and cannot be divided effectively between spatially disparate regions of a stimulus array^{2,3}, except under certain conditions^{7,8}. If visual arrays are scanned by serial movements of such a unitary attentional spotlight, then the search rate should be about the same whether the items are all presented to one visual hemifield or are equally distributed between the left and right hemifields. Alternatively, if each hemisphere in the split-brain patient controls its own attentional spotlight and can scan its respective hemifield independently of the other hemisphere, then the search rate should be faster for bilateral arrays than for unilateral arrays having the same set size. Presumably, intact interhemispheric communication should force normal subjects to maintain a single focus of attention for both unilateral and bilateral stimulus arrays⁹.

The performance of commissurotomy patients J.W. and L.B.,

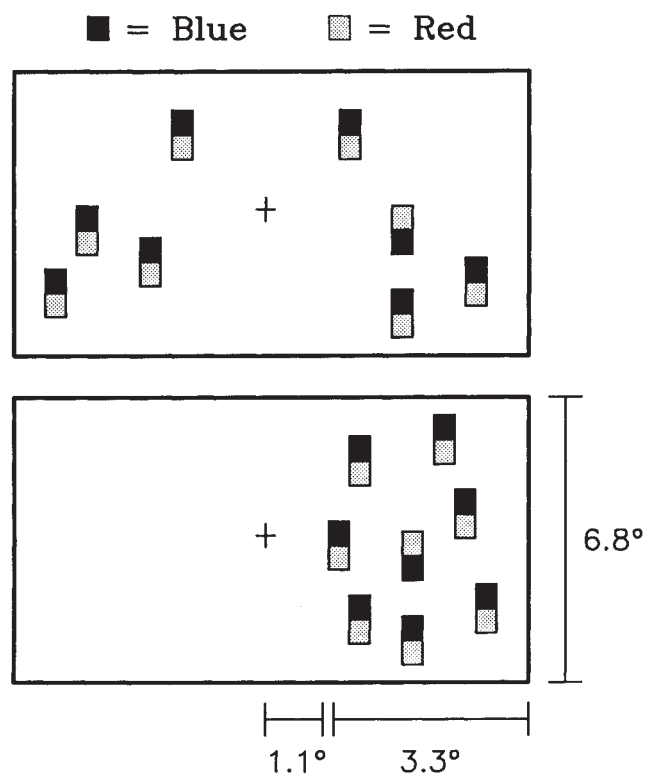


FIG. 1 Examples of stimuli used for the visual search task. The top panel is a bilateral array and the bottom panel is a unilateral array with the same set size (8 items). Each item subtended 0.4×0.8 degrees of visual angle. The items were placed in random positions within rectangular zones on the left and right sides; these zones measured 3.3×6.8 degrees and were displaced 1.1 degrees laterally from the fixation point. The arrays were presented for a duration of 2.5 s, with an interstimulus interval that varied randomly between 3.35 and 3.65 s. Unilateral and bilateral arrays of the various set sizes were randomly intermixed, and $\sim 50\%$ contained a target. Only one target could occur per trial. Each subject received a total of 792 trials.

whose case reports have been described elsewhere^{10,11}, was compared with a group of six neurologically normal young adults using the visual search paradigm shown in Fig. 1. The stimulus arrays consisted of rectangles constructed from a red square and a blue square, with blue on top for the distractors and red on top for the target. Subjects pressed a left-hand button if the target was in the left visual field (LVF), and a right-hand button if the target was in the right visual field (RVF); neither button was pressed on target-absent trials. Our design included set sizes 2, 4 and 8, presented either unilaterally or bilaterally (bilateral stimuli were divided equally between the LVF and RVF). Stimulus order was completely randomized. To ensure that the items were processed only by the contralateral hemisphere, subjects were instructed to fixate a central point at all times and eye position was verified by measurement of the electro-oculogram. For all patients and control subjects, horizontal eye movements averaged one degree of visual angle or less, which was smaller than the distance between the fixation point and the stimulus arrays.

The reaction time functions for the control and split-brain subjects are shown in Fig. 2. The control subjects' search functions were very similar for unilateral and bilateral arrays; reaction times were somewhat faster overall for bilateral arrays, but

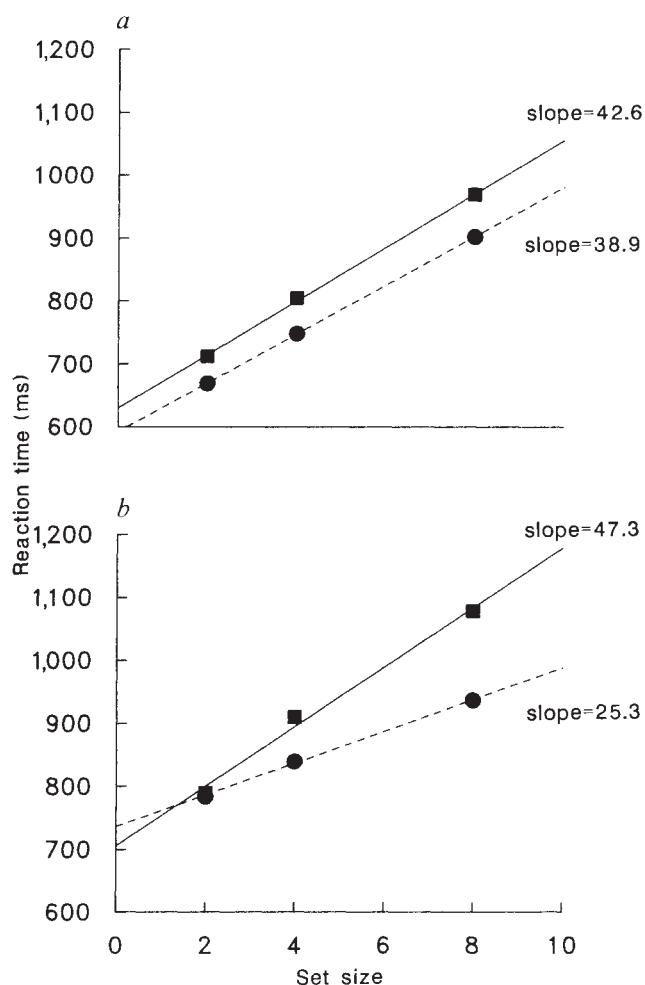


FIG. 2 Group mean reaction times as a function of set size for *a*, 6 normal control subjects and *b*, 2 split-brain patients. ■, Unilateral arrays; ●, bilateral arrays. In the control group, the search functions were essentially parallel for the unilateral and bilateral arrays, indicating a single focus of attention. For the split-brain patients, however, the slope was about twice as large for unilateral arrays as for bilateral arrays, suggesting that each hemisphere can search its corresponding hemifield independently of the other hemisphere.

there was no significant difference between the slopes of the reaction time functions for the two types of arrays (ANOVA, $P > 0.4$). For the split-brain patients, however, the slope of the search function was about twice as steep for the unilateral arrays as for the bilateral arrays. This 2:1 ratio of slopes would be expected if each hemisphere had an independent serial scanning mechanism, such that bilateral arrays were scanned with two spotlights and unilateral arrays were scanned with only one.

Multiple regression analyses of the single-subject reaction times confirmed that bilateral search slopes were significantly faster than unilateral search slopes for both split-brain patients ($P < 0.001$ for J.W.; $P < 0.04$ for L.B.), but not for any of the control subjects. Moreover, these differences were not due to speed/accuracy trade-offs: accuracy actually tended to be higher for bilateral arrays than for unilateral arrays, and this pattern was present for both the control group and for the split-brain patients (Table 1).

These findings indicate that the serial scanning of visual arrays for conjunction targets is conducted independently in the disconnected hemispheres of split-brain patients. Visual search can therefore be added to the list of perceptual and mnemonic processes that can be performed in parallel by the right and left hemispheres after commissurotomy¹². But our results stand in contrast to several previous studies indicating that attentional processes may be shared by the separated hemispheres through intact subcortical pathways^{13–15}. In particular, Holtzman and his colleagues reported that split-brain patients cannot divide the attentional spotlight in tasks that use symbolic cues to manipulate the direction of attention^{4,5}, which contrasts with our evidence that attention can be divided during visual search. These divergent results point to the existence of two functionally and anatomically distinct systems that participate in visual selective attention: whereas the separated cerebral hemispheres can function independently in scanning arrays of objects during visual search, the advance orientation of attention by means of a symbolic precue seems to be a joint exercise of the separated hemispheres, presumably mediated by intact subcortical structures.

This proposed dichotomy is consistent with studies of normal subjects¹⁶ showing that attentional orienting produced by symbolic precues does not improve the identification of feature conjunctions as effectively as the attentional processes deployed during visual search¹ or during the direct (peripheral) precuing of location^{17,18}. Our results are also in accord with earlier studies of split-brain subjects indicating that the two hemispheres can process information in parallel during simple binary decision^{9,19} and spatial memory tasks^{20,21}.

Although the search rates from the present experiment indicate that attention may operate independently in the disconnected hemispheres during the item-by-item search process, other aspects of the data provide evidence for some sharing of resources in this task. For the split-brain patients, the intercept of the search function was higher for bilateral arrays than for unilateral arrays, whereas the intercept was significantly lower for bilateral arrays in the control subjects. This finding indicates that increasing the processing load in one of the separated hemispheres may slow the overall target detection speed of the other hemisphere, although this interference does not affect the processing stage that conducts the serial target search. □

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Recovery of spatial learning deficits after decay of electrically induced synaptic enhancement in the hippocampus

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A WIDESPREAD interest in a long-lasting form of synaptic enhancement in hippocampal circuits^{1,2} has arisen largely because it might reflect the activation of physiological mechanisms that underlie rapid associative learning. As its induction normally requires the 'Hebbian'³ association of activity on a number of input fibres⁴, we refer to the process as long-term enhancement (LTE) rather than long-term potentiation (LTP), to emphasize its distinction from the ubiquitous, non-associative 'potentiation' phenomena that occur at most synapses, including those exhibiting LTP⁵. Among other evidence^{6–8} that LTE might actually have a role in associative memory is the demonstration that repeated high-frequency stimulation, which saturated the inducible LTE, caused a severe deficit in spatial learning, although it had no effect on well established spatial memory⁹. These results were consistent with a widespread view that information need only temporarily be stored in the hippocampal formation in order for long-term memories to be established in neocortical circuits^{10,11}. In this context, it is important to understand whether the possible underlying synaptic changes are of a permanent character, or are relatively transient. A second question is whether the actual cause of the observed learning deficit is the disruption of the synaptic weight distribution, and/or the limitation of further synaptic change, which presumably results from experimental saturation of the LTE mechanism. Alternatively, the deficit could be a consequence of some unobserved secondary effect of the high-frequency electrical stimulation. Here we demonstrate that learning capacity recovers in about the same time that it takes LTE to decay, which strongly favours the first possibility and supports the idea that LTE-like processes actually underlie associative memory.

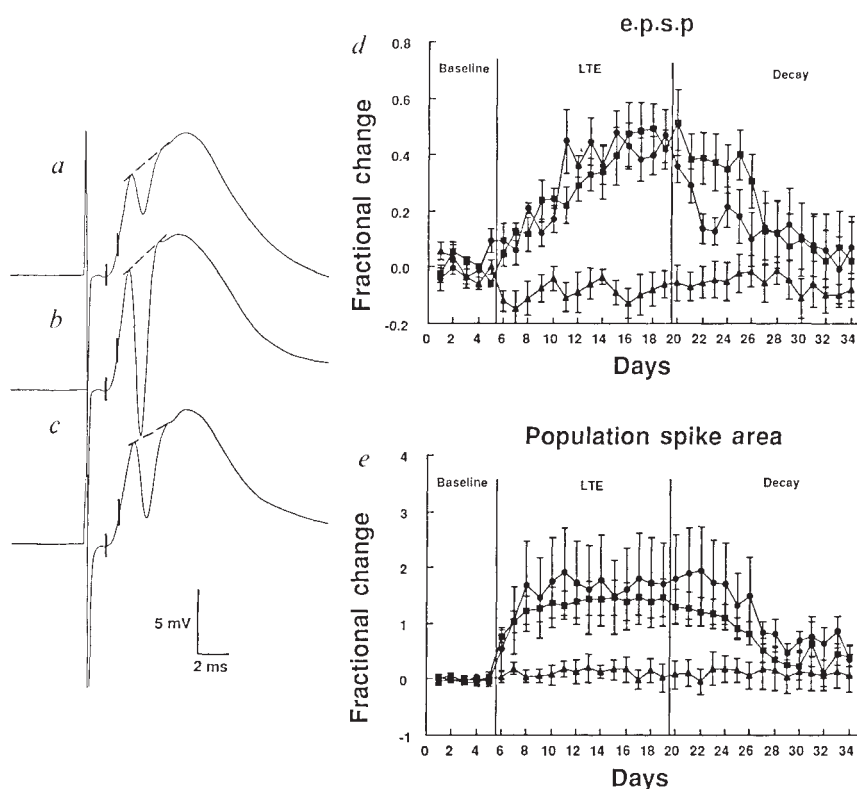
Experiments were performed on 12 male F-344 rats (10 months of age). Under deep sodium pentobarbital anaesthesia, all rats underwent bilateral implantation of electrodes⁹ for stimulation of perforant path fibres in the vicinity of the angular bundle and for recording the resulting synaptic and postsynaptic field potentials in the fascia dentata⁹ (see Fig. 1). Following recovery from surgery, the rats were randomly assigned to three groups. Two groups ($n = 4$) received high-frequency, LTE-inducing stimulation, and the other group ($n = 4$) received only low-frequency test stimuli. The electrical stimulation and physiological recordings were carried out daily over a 34-day period. The

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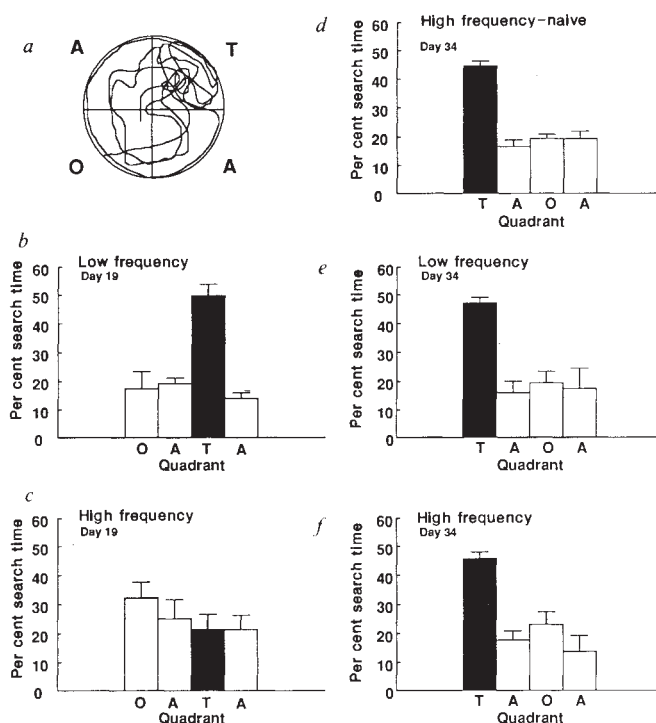
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FIG. 1 *a-c*, Representative examples of averaged extracellular field potentials recorded from the fascia dentata of conscious, unrestrained rats following single-pulse electrical stimulation of the major cortical afferents (perforant path) to the hippocampal formation. These responses were recorded during the baseline period (*a*), at the peak of long-term enhancement of the synaptic responses (*b*), which was induced by high-frequency stimulation, and following decay of the enhancement back to near-baseline levels (*c*). The tic marks indicate the positions on the rising phase of the synaptic component of the response between which the e.p.s.p. amplitude was measured. Postsynaptic discharge was assessed by measuring the area of the population spike underneath a tangent line (dashed line) joining the apparent onset and offset. *d* and *e*, Mean fractional change of the e.p.s.p. (*d*) and spike (*e*) components are plotted relative to the mean of the 5-day baseline period. The data for the three groups of animals are plotted over the 34 recording days. Two groups received high-frequency stimulation of the perforant path (squares and circles) each day between days 6 and 19 (indicated by vertical lines). The measures plotted during this period represent data recorded 24 h following each high-frequency session (that is, immediately before the current day's high-frequency session). The other group received low-frequency test stimuli only (triangles). In the high-frequency (HF) groups, both the e.p.s.p. and population spike components were increased substantially as a result of the high-frequency stimulation. These increases reached asymptotes by day 19, the end of the 14-day high-frequency period. During the next 15 days, the increases decayed back almost to control levels. Note that the responses for the low-frequency group (LF) remained relatively stable throughout the recording period, thus demonstrating that the decay of LTE is not due to possible deterioration of the recording preparation. Consistent with this description, a two-way analysis of variance on the e.p.s.p. amplitude data revealed significant effects of Groups (HF versus LF versus HF-naive), $F(2, 19)=10.9$, $P<0.01$; Days (Days 1–34), $F(33, 627)=5.0$, $P<0.01$; and



Groups $\times$ Days interaction, $F(66, 627)=2.4$, $P<0.01$. Two-way analysis of variance of the population spike area data revealed significant effects of Days, $F(33, 627)=6.7$, $P<0.01$ and Group $\times$ Days interaction, $F(66, 627)=1.5$, $P<0.01$. Post hoc analysis (Newman-Keuls, $P<0.05$) of the interactions for both the e.p.s.p. amplitude and population spike area showed that: (1) on day 19 the LF group differed from the two HF groups; (2) on day 19 the two HF groups did not differ from each other; and (3) on day 34 there were no differences among any of the groups.

FIG. 2 *a*, A typical search pattern during the 'probe' trial of a rat that had previously learned the spatial location of the escape platform in the Morris water task. During the probe trial, the platform was removed from the pool, and the animal was placed into the water and allowed to search the pool for 60 s. For purposes of analysis, the apparatus was divided into four quadrants: T, training quadrant that contained the escape platform; A, adjacent quadrant; and O, opposite quadrant. The amount of time each animal spent in each quadrant was recorded. *b* and *c*, Mean percentage of time spent in each of the four quadrants for the LF and HF groups on day 19 following experimentally induced saturation of LTE in the fascia dentata of the HF group. A one-way analysis of variance of quadrant search time for the LF group was significant, $F(3, 9)=13.3$, $P<0.01$. Post hoc analysis (Newman-Keuls, $P<0.01$) showed that the animals in the LF group spent significantly more time in the training quadrant compared with any of the other quadrants. *d-f*, The mean percentage of time spent in the four quadrants for the HF behaviourally naive group, the LF control group, and the HF group that was previously trained on day 19. On this day, the platform was moved to the location diagonally opposite its day-19 location. These data show that the behavioural impairment associated with LTE saturation recovers with the decay of LTE. Separate one-way analyses of variance on these data were significant, all F values $(3, 9)>8.3$, P values <0.01 , and post hoc analyses (Newman-Keuls, $P<0.01$) showed that for all groups all the rats spent more time in the training quadrant than in any of the other quadrants.



first 5 days of this period served as a baseline test, during which responses were elicited alternately in each hemisphere at 5-second intervals using single electrical stimuli⁹. Thirty responses were collected from each hemisphere each day. During the next 14 days, high-frequency stimulus bursts (10 pulses at 400 Hz) were substituted for the low-frequency test pulses on cycles 11 through to 20 of the test session for each hemisphere of the two high-frequency groups. During the final 15 days, all groups received the same low-frequency test stimulation. The measures of synaptic strength and postsynaptic activation over this period are illustrated in Fig. 1. Whereas these responses were relatively stable throughout the observation period in the low-frequency group, the high-frequency groups showed a substantial increase in both the excitatory postsynaptic potential (e.p.s.p.) and population spike components. These increases reached asymptote within the 14-day high-frequency period¹² and decayed back to baseline over the course of the subsequent 15-day test period.

Behavioural tests of spatial learning capability were carried out on days 19 and 34. At the peak of LTE-saturation on day 19, the low-frequency group and one of the high-frequency groups of rats underwent training. The other high-frequency group was not trained until day 34. The behavioural apparatus was a Morris water pool, located in a room that contained numerous distal visual cues. The rats were trained to escape to a platform hidden in a fixed position just beneath the surface of the water. A training session consisted of 6 blocks of 2 trials, each block separated by intervals of 2 min. Immediately following the last training trial, each rat was given a 60-second 'probe' trial, during which the platform was removed from the pool. The relative amount of time that the rat spent searching in the training quadrant served as a measure of spatial learning (Fig. 2). Latency to escape from the water during training served as an additional measure (Fig. 3). To control for possible motor or sensory deficits, after the probe trial we assessed the rats' ability to escape to a visible platform located in the original training quadrant. On day 34, following LTE decay, all three groups of rats were trained to find the platform in a location diagonally opposite to the day-19 location. The high-frequency group that received no training on day 19 served as behaviourally naive controls for the test of learning following LTE decay.

At the peak of LTE saturation, the high-frequency rats were severely impaired at spatial learning, as indicated by their failure to show differential quadrant search during the probe trial and their longer escape latencies during training. There were no between-group differences in escape latency on the visible platform test. Following the decay of LTE, there were no differences among groups in either quadrant search time or escape latencies, with the exception of a slight reduction in the escape latency on the first trial in the control group, which presumably reflected some savings of the procedural aspects of the task. Two other measures, heading error and latency to first annulus crossing (data not shown), also revealed comparable between-group differences on day 19 and lack of differences on day 34. These data thus show (1) that LTE in the primary input stage to the hippocampal formation is not permanent, and (2) that the spatial-learning impairment that accompanies LTE saturation recovers when experimentally induced LTE decays back to near-baseline levels.

Assuming that experimentally induced LTE in fact reflects the activation of the main mechanism for information storage in the fascia dentata (an assumption that is not yet proven), these results support the general hypothesis that spatial memory involves an initial, temporary synaptic encoding in the fascia dentata of information, some of which may be destined for more permanent storage at subsequent processing stages. Taken in conjunction with the findings of Staubli *et al.*¹³, that LTE induced under comparable conditions in CA1 synapses does not show appreciable decay over a comparable time course, our results indicate that factors regulating the rate of decay of LTE may

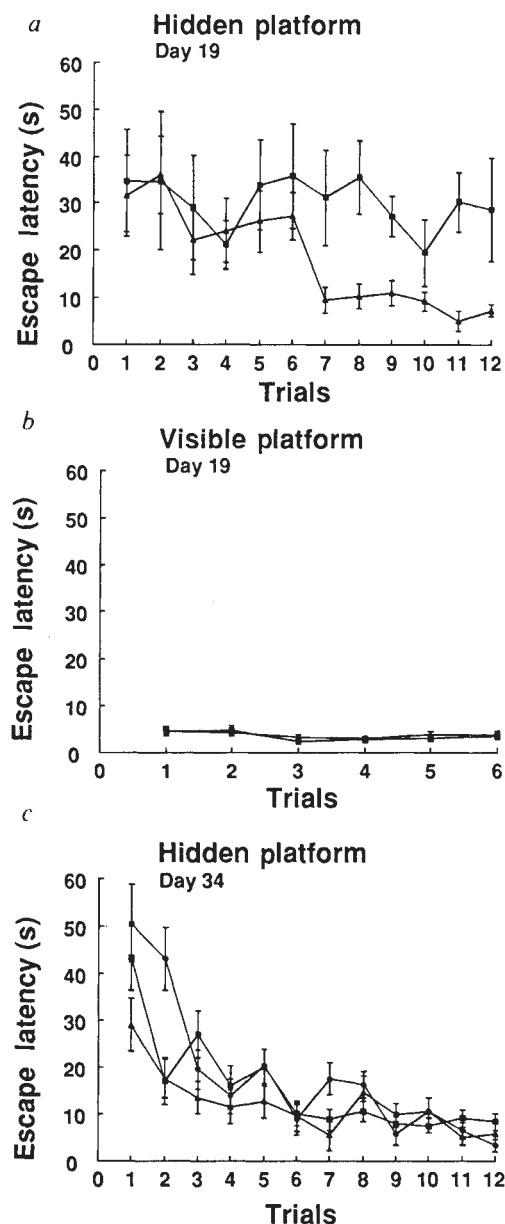


FIG. 3 a, Mean escape latencies as functions of trials for low- and high-frequency groups at the peak of LTE saturation on day 19. Whereas the LF rats reduced their escape latencies over trials on the hidden platform task, the HF group showed no such improvement (main effect for Groups $F=5.6$ (1, 6) $P<0.05$). There were no differences between groups, however, in latency to escape to a visible platform (b). c, Mean escape latencies as functions of trials following decay of LTE on day 34. There were no differences on the hidden platform task among the LF control and the two HF groups. Thus the escape latency data also support the conclusions that the learning impairment on day 19 is due to saturation of LTE and that the impairment recovers as LTE decays. ▲, Low-frequency group; ■, high-frequency group; ●, high frequency-naïve control group.

exhibit significant regional variation in the nervous system. Whether this decay is due to some passive process or is regulated by ongoing neural activity remains a significant open question. □

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Induction of glia-derived nexin after lesion of a peripheral nerve

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GLIA-derived nexin¹ (GDN), also known as protease nexin^{1,2,3}, is a serine protease inhibitor of deduced relative molecular mass 41,700, identified in conditioned media of glioma cells by its neurite-promoting activity⁴. GDN can promote neurite outgrowth *in vitro* from neuroblastoma cells^{5,6}, sympathetic neurons⁷ and hippocampal neurons (L. Farmer *et al.*, manuscript in preparation). *In vivo*, GDN is constitutively expressed in all parts of the olfactory system⁸, where axonal regeneration and neurogenesis occur continuously throughout life. This observation indicates that GDN could be important for axonal regeneration *in vivo*. To investigate this possibility, we have taken advantage of the fact that damage to nerves in the peripheral nervous system leads to their regeneration, whereas in the central nervous system no such regeneration can occur. Here we report that after lesion of the rat sciatic nerve there is a large transient increase in the amount of GDN messenger RNA and of released GDN. The cells showing GDN immunoreactivity are mainly localized distal to the lesion site. These results further support the suggestion that GDN is important for axonal regeneration *in vivo*, and indicate that protease inhibitors could have a role in Wallerian degeneration and peripheral nerve regeneration.

Analysis of northern blots of total RNA from whole rat sciatic nerve showed that crushing the nerve induced the synthesis of GDN mRNA; this induction reached a maximum six days after injury (Fig. 1). In sham-operated nerves, exposed but without injury, GDN transcript was not induced. The induction was about sevenfold greater 6 days after lesion than it was 2 days after lesion. The kinetics of the release of GDN protein from a 5-mm nerve segment taken just distal to the lesion site were similar but delayed compared with those of the GDN RNA pool in the whole nerve. GDN was maximally induced seven days after lesion, and from then on its level declined (Table 1). One and a-half days after injury, the amount of GDN released from this small nerve segment was indistinguishable from that released from control tissue derived from an intact nerve. Immunoblot experiments with homogenized tissue confirmed that the maximum release of GDN was seven days after lesion (Fig. 2). Furthermore, the elevated levels of GDN were not

TABLE 1 Release of GDN from nerve segments removed at different times after crushing the sciatic nerve

Days after injury	Injured nerve (ng GDN per 10 mg dry weight)	Contralateral control nerve (ng GDN per 10 mg dry weight)	No. of fold increase in GDN release
1.5	1.2	1.4	1.2
7	6.8	1.8	3.8
7*	5.7	1.5	3.8
10	3.7	1.1	3.4
14	1.4	0.99	1.4

At the indicated days after crushing the sciatic nerve, a 5-mm segment just distal to the lesion site was removed. For each time point, 10 animals were killed. The nerve pieces were incubated for 3 h in modified Eagle's medium containing 1 mg ml⁻¹ bovine serum albumin. The amount of GDN diffusing out of the tissue was determined by an ELISA.

* The GDN determination for day 7 was repeated in a totally independent experiment.

correlated exclusively to axonal regeneration, because the induction of GDN was even observed after transecting the nerve (Fig. 2).

Immunohistochemical studies were performed to detect lesion-induced GDN synthesis at the cellular level. Only a few cells were stained weakly positive in sections of uninjured nerve (Fig. 3f). Both the intensity and the number of cells stained by anti-GDN antibodies were greater in nerves four (data not shown) and seven days after injury than in contralateral intact control nerve (Fig. 3; a and c compared with d). These strongly stained cells were not predominantly localized around the lesion

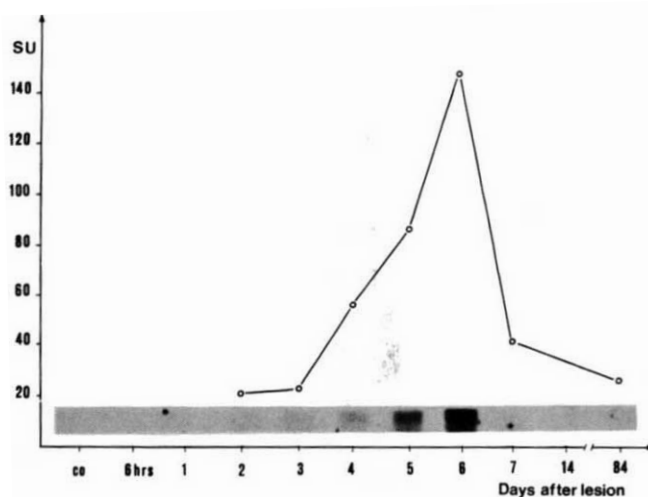


FIG. 1 Transient increase in the steady-state level of GDN RNA after crushing sciatic nerves. The GDN transcript was not detectable in uninjured control nerves (co), 6 h, and 1 day after lesion. The induction as estimated by densitometry 6 days after lesion was about sevenfold greater than that 2 days after the lesion. On day 7, GDN-RNA was already reduced to undetectable levels.

METHODS. Nerves of Wistar rats were crushed as previously described²² and analysed after 6 h, 1, 2, 3, 4, 5, 6 and 7 days, 2 weeks and 3 months. The distal segments of 20 nerves were surgically removed and RNA prepared by the guanidine isothiocyanate method²³. Total RNA (8 µg) was fractionated on a urea-agarose gel and transferred to Nytran NY 13 membrane. The filter was prehybridized (50% formamide, 5×Denhardt's solution, 0.1% SDS, 5×SSPE (1×SSPE: 150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4), 200 µg ml⁻¹ salmon sperm DNA, 10 µg ml⁻¹ *Escherichia coli* DNA, 100 µg ml⁻¹ poly(U) at 42 °C for 4 h and hybridized overnight in the same solution with random-primed [³²P]dCTP-labelled rat GDN complementary DNA consisting of the large *Pst*I restriction fragment of the recombinant G2 clone²⁴. The blot was washed twice, each time for 30 min at 65 °C in 0.1×SSC, 1% SDS, exposed to X-ray film and scanned densitometrically by introducing arbitrary scan units (SU).

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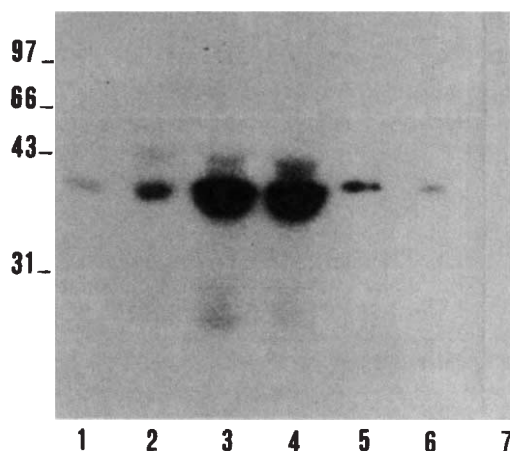


FIG. 2 After lesion, the amount of GDN increases in sciatic nerve tissue. Low amounts of GDN could be detected by western blotting in contralateral uninjured control nerves (lane 1) and sham-operated animals (lane 2). Crushed and transected nerves contained similar higher amount of GDN (lanes 3 and 4) 7 days after injury. The detection limit of the immunoblot was about 25 ng GDN, as judged by immunoblotting 50 ng and 25 ng of purified GDN (lanes 5 and 6, respectively). Lane 7 represents a negative control using a nonspecific monoclonal antibody and the same concentration of iodinated anti-mouse IgG.

METHODS. Nerves were removed and homogenized in lysis buffer⁸ using a Polytron homogenizer at 7,000 r.p.m. for 1 min. The crude homogenate was centrifuged at 18,000 r.p.m. in a SS34 rotor for 15 min and concentrated 2–4-fold by an Amicon microconcentrator. The homogenate was again centrifuged as described above. Total protein (50 µg) was separated by SDS-PAGE according to Laemmli and analysed by immunoblotting, using a mixture of two monoclonal antibodies 4B3 and 2D5 raised against purified GDN.

site, but were irregularly scattered throughout the distal nerve segment and could not be detected proximal to the lesion site where the nerve fibres did not degenerate. Proximal to the lesion site, the intensity of immunostaining was similar to that of the uninjured control nerve.

In this *in vivo* model, the up-regulation of GDN coincided with infiltration of macrophages recruited in significant numbers in the first 3–5 days after the injury⁹. Double-immunofluorescent

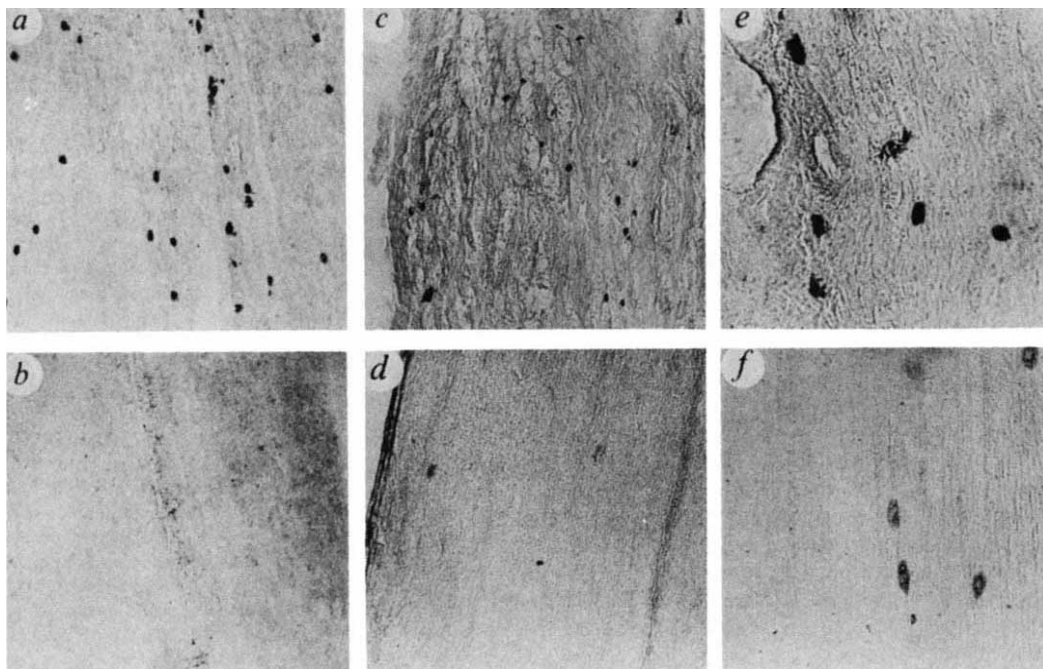
studies showed that the GDN-synthesizing cells did not express the typical cytoplasmic ED 1 marker for macrophages¹⁰, nor were these cells positive for Thy-1 (data not shown), an antigen which in rodents can be used as a marker for fibroblasts¹¹. Macrophages could also regulate GDN synthesis indirectly, by eliciting a stimulatory molecule. For example, interleukin-1 (IL-1), secreted by activated macrophages¹², induces the transcription of nerve growth factor *in vitro*¹³, which is another molecule that is up-regulated after a peripheral nerve lesion¹⁴; experiments with sciatic nerve explant cultures, however, show that IL-1 does not significantly increase the release of GDN (data not shown). But these data do not completely rule out a physiological function of macrophages in regulating GDN synthesis *in vivo*.

The S-100 antigen characteristic of Schwann cells was also not convincingly co-localized with the small number of GDN-producing cells, although cultured Schwann cells derived from sciatic nerves from 3–4-day-old rats strongly express GDN (A. Bleucl, personal communication). In tissue sections of freshly dissected nerves from these animals, few GDN-positive cells could be demonstrated. Therefore GDN production is apparently induced by cultivating these cells. These data indicate, considering the tremendous cell proliferation during axonal regeneration, that a small subpopulation of Schwann cells or progenitor cells could exist, which can be stimulated by a mediator molecule(s) to produce GDN *in vivo* but which lack markers specific for mature cells.

So far the physiological function of GDN during axonal regeneration in the olfactory tract and the sciatic nerve has not been established. The up-regulation of GDN described here occurs later than the increase in the steady-state level of the mRNAs coding for nerve growth factor (NGF) and the receptor for NGF¹⁴. The appearance of GDN could thus be associated with its permissive effect on neurite outgrowth. Alternatively, GDN could prevent proteolytic degradation of re-expressed cell adhesion molecules such as NgCAM, N-CAM (ref. 15) and/or could protect the extracellular matrix¹⁶ from proteases such as plasminogen activator¹⁷. This protective function of GDN could be possible because GDN itself as well as plasminogen activator inhibitor 1 are localized in the extracellular matrix of cultured fibroblasts^{18,19}. This immobilization to the cell surface²⁰ or to the matrix apparently changes the inhibitory specificity of GDN because thereafter only thrombin, but not urokinase or plasmin,

FIG. 3 GDN positive cells were detected in the distal nerve stump after transecting (a) and after crushing the nerve (c) using specific anti-GDN IgG⁸. The specificity of the immunostaining was shown by incubating longitudinal sections of transected nerve tissue with preimmune serum (b). (d), Intact control nerve processed parallel to the nerve of c. The staining of cells for GDN is more pronounced in injured (e) than in intact (f) nerve.

METHODS. Rat sciatic nerves were transected or crushed. Seven days after lesion the nerve was removed and embedded without fixation in Tissue-Tek. Cells expressing GDN were detected by using the Vectastain system²⁵.



can be inhibited²¹. Whether this change in the specificity of GDN has consequences for events in axonal regeneration or whether it is why GDN needs to be resynthesized after a nerve injury, is at the moment a matter of speculation. □

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Modulation of dihydropyridine-sensitive Ca^{2+} channels by glucose metabolism in mouse pancreatic β -cells

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GLUCOSE stimulates insulin secretion from the pancreatic β -cell by increasing the cytosolic calcium concentration¹. It is believed that this increment results mainly from Ca^{2+} influx through dihydropyridine-sensitive calcium channels because insulin secretion is abolished by dihydropyridine antagonists² and is potentiated by dihydropyridine agonists³. Glucose may influence Ca^{2+} influx through these channels in two ways: either by regulating the β -cell membrane potential or by biochemical modulation of the channel itself. The former mechanism is well established. Glucose metabolism, by closing ATP-sensitive K^{+} channels⁴, depolarizes the β -cell membrane and initiates Ca^{2+} -dependent electrical activity, with higher glucose concentrations further increasing Ca^{2+} influx by raising the frequency of action potentials^{5,6}. We show here that glucose metabolism also increases calcium influx directly, by modulating the activity of dihydropyridine-sensitive Ca^{2+} channels.

Figure 1 illustrates the effect of glucose on the whole-cell Ca^{2+} currents, recorded under physiological ionic conditions using the perforated patch configuration of the patch clamp method⁷, which retains metabolic and second messenger systems intact. Depolarization to 0 mV elicits an inward Ca^{2+} current which has properties similar to those recorded using conventional whole-cell methods^{8,9}. Addition of 20 mM glucose to the

bath solution produces a roughly 2-fold increase in the peak Ca^{2+} current.

The whole-cell Ca^{2+} current is the product of the single channel current (i), the number of functional Ca^{2+} channels in the cell membrane (N), and the channel open probability (P). To determine which of these parameters is increased by glucose we have recorded Ba^{2+} currents flowing through single Ca^{2+} channels in cell-attached patches on intact β -cells. As previously described¹⁰, most patches contained only a single type of Ca^{2+} channel with properties characteristic of L-type Ca^{2+} channels¹¹.

The effect of glucose on the L-type Ca^{2+} channel currents is illustrated in Fig. 2. In the absence of the sugar, channel activity was constant for periods of up to 10 min and was unaffected by perfusion. Addition of 20 mM glucose to the bath solution produced an increase in channel activity which was evident within 2 min of the start of perfusion but often took several minutes to reach a steady-state. Channel activity (defined as NP) was increased 3-fold by 20 mM glucose from 0.007 ± 0.004 to 0.024 ± 0.012 ($n = 5$).

In the absence of glucose, channel openings were infrequent and of brief duration. Open times could be fitted by a single

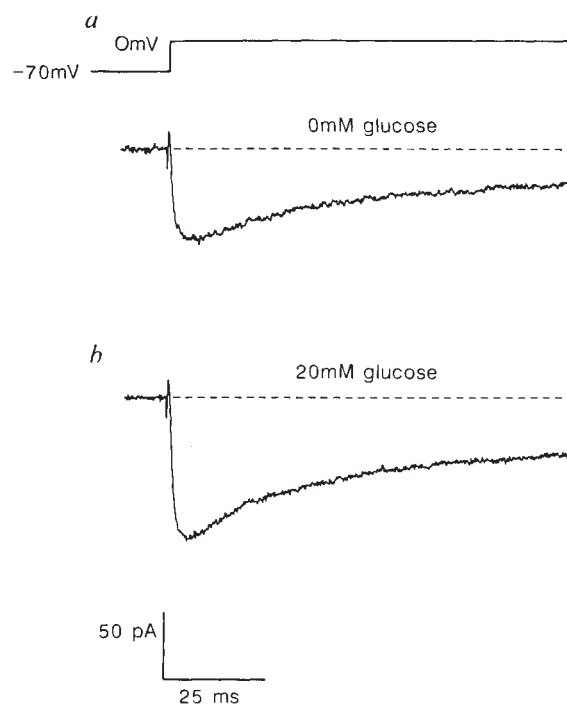


FIG. 1 Effect of glucose on whole-cell Ca^{2+} -currents. Membrane currents recorded in response to a depolarizing voltage step to 0 mV from a holding potential of -70 mV in glucose-free solution (a) and about 6 min after the addition of 20 mM glucose to the bath (b). The dashed line indicates the zero current level. Currents are leak-corrected. Sample rate, 13 kHz; records filtered at 5 kHz; cell capacitance 5.5 pF; access resistance 50–60 M Ω ; temperature 25 °C.

METHODS. Mouse pancreatic β -cells were isolated and maintained in primary tissue culture as described previously¹⁰. Cells were pre-incubated for 20 min at room temperature in normal external solution without glucose before beginning experiments: 138 mM NaCl, 5.6 mM KCl, 10 mM TEACl, 1.2 mM MgCl_2 , 2.6 mM CaCl_2 , 10 mM HEPES-NaOH, pH 7.4. Whole-cell currents were recorded using the perforated patch configuration of the patch clamp method⁷ in which the patch membrane is permeabilized by the pore-forming antibiotic nystatin. The pipette solution contained: 10 mM NaCl, 10 mM KCl, 70 mM Cs_2SO_4 , 7 mM MgCl_2 , 10 mM HEPES-NaOH, pH 7.4 and 50 $\mu\text{g ml}^{-1}$ nystatin (in 0.1% dimethylsulphoxide). Cs^{+} was used as an impermeant K^{+} -substitute to reduce voltage-activated outward K^{+} -currents. The holding potential was -70 mV and pulses were applied at a frequency of 0.1 Hz. Currents were recorded using a List-electronic EPC7 patch clamp amplifier and stored on video tape. Exchange of the bath solution was complete within 2 min.

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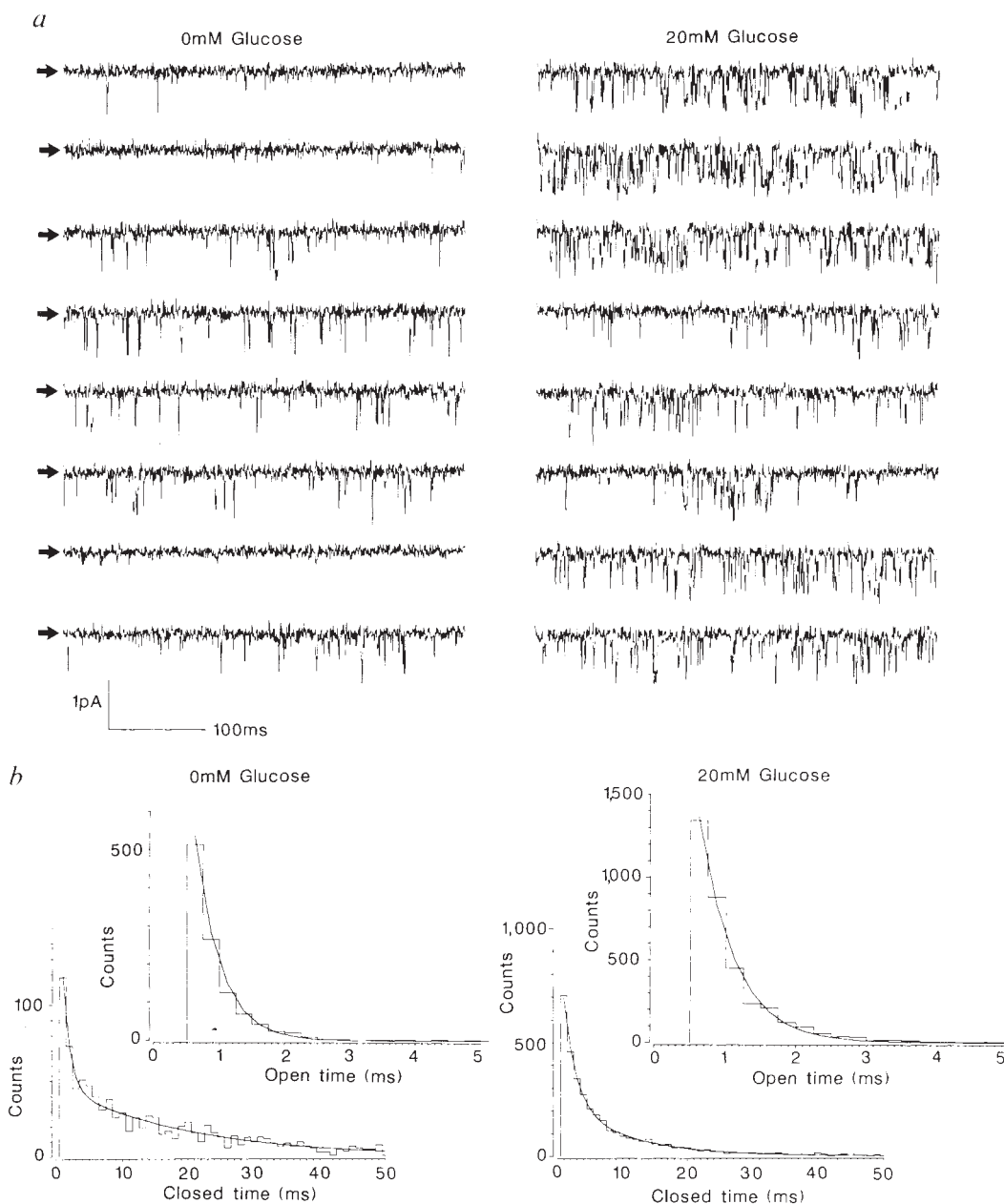
exponential (with a time constant of 0.35 ± 0.03 ms; $n = 5$) and closed times were best fitted by the sum of two exponentials (fast time constant, 1.8 ± 0.4 ms; slow, 25.8 ± 8.2 ms; $n = 5$). In 20 mM glucose, the mean open time was barely altered (0.44 ± 0.04 ms; $n = 5$). The fast component of closed times was also unchanged (1.3 ± 0.15 ms; $n = 5$), but there was a 2-fold decrease in the apparent time constant of the slow component of closed times (to 12.0 ± 3.3 ms; $n = 5$). In addition, the number of null sweeps decreased from $36.4 \pm 14\%$ to $21.8 \pm 5.5\%$. But we

acknowledge that we are unable to discriminate an effect on the slow component of closed times from the recruitment of additional Ca^{2+} channels. In these experiments, the open probability was not high enough to enable us to establish the number of channels in the patch and thus distinguish an effect on P from one on N .

One problem we encountered in quantifying the effect of glucose on Ca^{2+} channel currents was the low frequency of Ca^{2+} channel openings and their very short lifetimes. Better resolution

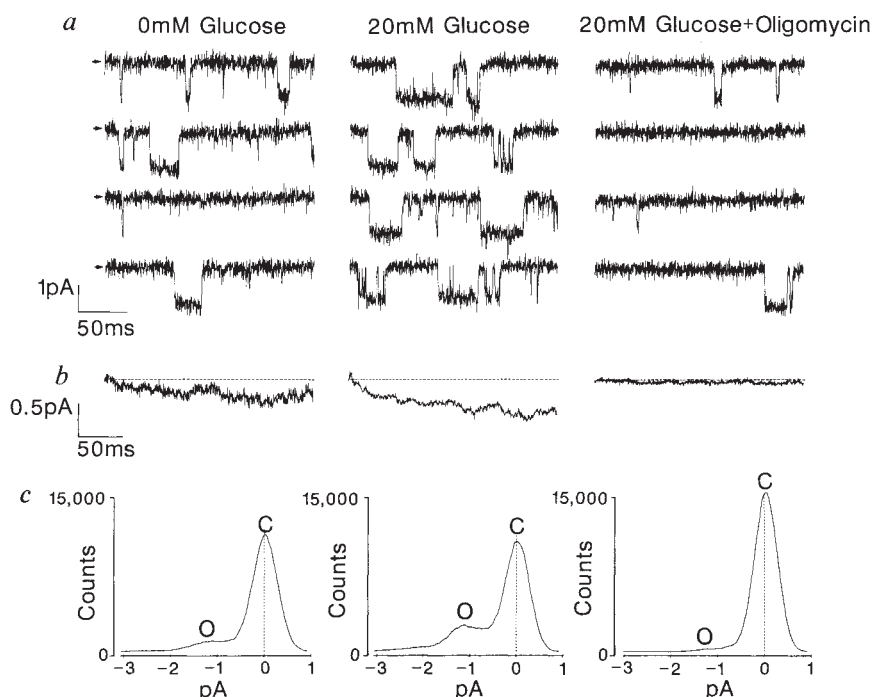
FIG. 2 Glucose increases the activity of single Ca^{2+} -channel currents. *a*, Eight examples of single channel currents recorded from a cell-attached patch before (left) and about 4 min after (right) the addition of 20 mM glucose to the bath solution. The membrane potential was stepped to 0 mV from a holding potential of -70 mV. The arrows to the left of each record indicate the current level when the channels were closed. Sample rate, 4 kHz; records filtered at 2 kHz. *b*, Distribution of open and closed times for the same patch as in *a* recorded in 0 mM glucose (left) and 20 mM glucose (right) during a depolarization to 0 mV. Open times: Bin size displayed, 250 μs . Open times were fit by a single exponential function $f(t) = A \cdot \exp(-t/t_0)$ using a least squares algorithm. Zero glucose, $A = 1,932$, $t_0 = 0.4$ ms; 20 mM glucose, $A = 3,708$, $t_0 = 0.5$ ms. Closed times: Bin size displayed, 1 ms. Closed times were best fit by the sum of two exponentials: $f(t) = A \cdot \exp(-t/t_{c1}) + B \cdot \exp(-t/t_{c2})$ using a least squares algorithm. Zero glucose, $A = 156$, $t_{c1} = 1.1$ ms, $B = 48$, $t_{c2} = 19.8$ ms; 20 mM glucose, $A = 736$, $t_{c1} = 1.8$ ms, $B = 256$, $t_{c2} = 10$ ms. Number of closed times longer than 50 ms, 154 (0 mM glucose), 130 (20 mM glucose).

METHODS. For single channel recording, cells were pre-incubated in a modified Krebs-Ringer bicarbonate-buffered external solution without glucose at 37°C for more than 30 min before beginning experiments: 103 mM NaCl, 25 mM NaHCO_3 , 1.2 mM NaH_2PO_4 , 4.7 mM KCl, 2.6 mM CaCl_2 , 1.1 mM MgCl_2 , 5% CO_2 , pH 7.4. The pipette solution contained: 100 mM BaCl_2 , 10 mM TEACl, 10 mM HEPES- $\text{Ba}(\text{OH})_2$, pH 7.4. In normal external solution (5.6 mM KCl), cells fired action potentials in response to glucose. To control the β -cell membrane potential therefore, cells were bathed in a depolarizing high K^+ -solution containing: 115 mM KCl, 28 mM KOH, 1 mM MgCl_2 , 1 mM CaCl_2 , 10 mM EGTA (free Ca^{2+} , $0.08 \mu\text{M}$) and 10 HEPES (pH 7.4); in some experiments (Figs 3, 4) $0.1 \mu\text{M}$ BAYK8644 was added to increase channel open times and facilitate measurements. Experiments were carried out at 22 – 24°C . The holding potential was -70 mV and pulses were applied at a frequency of 0.5 Hz. Currents were later replayed through an 8-pole



Bessel filter (-3 dB at 2 or 3 kHz), digitized at 4 or 8 kHz (in the absence and presence of BAYK8644 respectively) and analysed by computer. Capacity and leakage currents associated with potential steps were removed by averaging those records that contained no opening and subtracting this average from the records. In most patches we observed only the dihydropyridine-sensitive type of single Ca^{2+} channel current. In a few cases, however, long-lasting currents were observed which reversed at potentials positive to 20 mV and which had a single channel slope conductance of 6 pS (100 mM Ba^{2+}). Channel openings occurred throughout the pulse and were not markedly voltage-dependent, suggesting this channel is distinct from the T-type Ca^{2+} channel. We have not included such recordings in our analysis.

FIG. 3 The effect of glucose depends on cell metabolism. *a*, Single channel currents recorded from a cell-attached patch in glucose-free solution (left), about 8 min after the addition of 20 mM glucose to the bath solution (centre) and 8 min after the subsequent addition of 20 mM glucose plus 2.4 $\mu\text{g ml}^{-1}$ oligomycin (right). The membrane potential was stepped to -20 mV from a holding potential of -70 mV . Sample rate, 8 kHz; records filtered at 3 kHz. This patch contained at least 2 channels as indicated by 2 simultaneous openings. All records (*a*, *b*, *c*) were obtained in the presence of 0.1 μM BAYK8644. *b*, The associated mean currents obtained by averaging 100 traces in 0 mM glucose (left), 20 mM glucose (centre) and 20 mM glucose plus 2.4 $\mu\text{g ml}^{-1}$ oligomycin (right). The steady-state value of the mean current was 0.166 pA in 0 mM glucose, increased to 0.34 pA in 20 mM glucose and was reduced to 0.035 pA in 20 mM glucose plus oligomycin. The dashed line indicates the zero current level. *c*, Amplitude histograms recorded in 0 mM glucose (left), 20 mM glucose (centre) and 20 mM glucose plus 2.4 $\mu\text{g ml}^{-1}$ oligomycin (right). 'C' and 'O' correspond to the closed and first open levels respectively. Note that the single channel current amplitude does not change ($\sim 1.1\text{ pA}$). The dashed line indicates the zero current level.



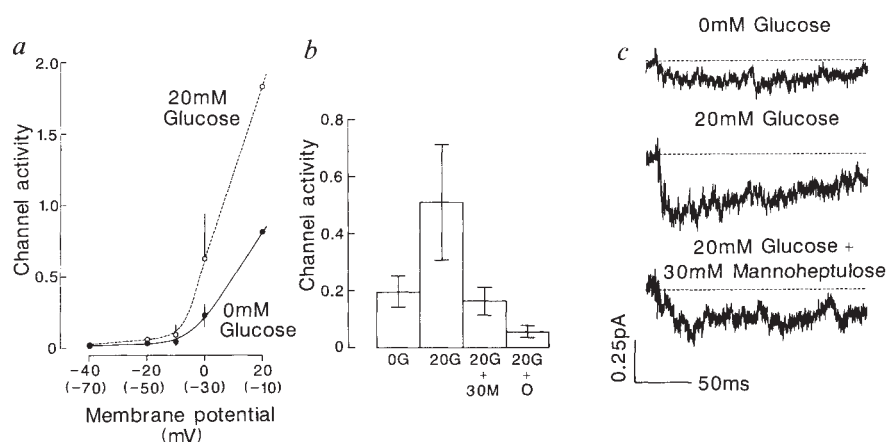
of Ca^{2+} -channel currents was obtained in the presence of the dihydropyridine agonist BAYK8644 and the effects of glucose were qualitatively similar, leading to an increase of the average current of 3.05 ± 1.08 fold ($n = 11$). In these experiments, it was clear that glucose had no effect on the unitary current amplitude (Fig. 3*a*, *c*); thus the increase in the whole-cell currents produced by glucose must be the result of an increase in N or P , or both. Even in the presence of BAYK8644, the open probability was still not great enough for us to determine whether glucose affected N and thus whether the principal effect of the sugar is on P or on N .

Figure 4*a* shows that glucose increases NP over a range of membrane potentials: under physiological ionic conditions, the relationship between membrane potential and NP will be shifted by $\sim 30\text{ mV}$ to more negative potentials (see legend). Importantly, channel activity (and thus Ca^{2+} influx) became significant at potentials that failed to elicit channel openings in the absence

of glucose, suggesting the glucose lowers the threshold for β -cell electrical activity. The greatest increase in NP was found at potentials positive to -35 mV , where action potentials and slow waves are elicited, suggesting that glucose markedly potentiates electrical activity and Ca^{2+} influx. Although the effect reported here of glucose on Ca^{2+} -channel kinetics in normal β -cells resembles that of glyceraldehyde on Ca^{2+} channels in the insulin-secreting β -cell line RINm5F (ref. 12), the effect on the voltage-dependence of channel activity is somewhat different.

Our results also indicate that, like the effects of glucose on insulin secretion¹³ and ATP-sensitive K^{+} -channels⁴, the metabolism of the sugar is required for stimulation of Ca^{2+} channel activity. First, as glucose was applied to the bath solution and not directly to the membrane patch, the sugar must mediate its effect through an intracellular route. Secondly, mannoheptulose (30 mM; Fig. 4*b*, *c*), a specific inhibitor of glucose metabolism¹³, was capable of reversing the effect of glucose on Ca^{2+} -channel

FIG. 4 *a*, Voltage dependence of channel activity (NP) in 0 mM glucose (filled circles) and in 20 mM glucose (open circles). The steady-state level of NP was measured by averaging 100 test pulses to the indicated potential in each solution. The data points indicate the mean of 2–7 samples and the vertical lines indicate one s.e.m. All measurements were made in the presence of 0.1 μM BAYK8644. The use of 100 mM Ba^{2+} in the pipette shifts the voltage-dependence of the open probability by 30 mV to more positive potentials, relative to that in 2.6 mM Ca^{2+} solution. This is due to a more extensive screening of membrane surface charge. The potentials after correction for surface charge are shown in parentheses. *b*, Channel activity recorded at 0 mV for the same 11 patches in 0 mM glucose (left, 0 G) and in 20 mM glucose (centre left, 20 G), and subsequently for 4 of these patches in 20 mM glucose plus 30 mM mannoheptulose (centre right, 20 G + 30 M) and for 4 of the other patches in 20 mM glucose plus 2.4 $\mu\text{g ml}^{-1}$ oligomycin (right, 20 G + O). Vertical bars indicate one s.e.m. *c*, Mean currents recorded in response to a depolarizing voltage step to 0 mV from a holding of -70 mV in glucose-free solution (top), about 6 min after the addition of 20 mM glucose to the bath (middle) and after 6 min in 20 mM glucose plus 30 mM mannoheptulose. Mannoheptulose



blocks glycolysis at the level of hexose phosphorylation. The dashed line indicates the zero current level. Mean currents were obtained by averaging 69 traces in 0 mM glucose, 76 traces in 20 mM glucose and 50 traces in 20 mM glucose plus mannoheptulose. The steady-state value of the mean current was 0.08 pA in 0 mM glucose, increased to 0.25 pA in 20 mM glucose and was reduced to 0.13 pA in 20 mM glucose plus mannoheptulose.

activity. Furthermore, oligomycin ($2.4 \mu\text{g ml}^{-1}$; Figs 3 and 4b), a potent inhibitor of oxidative phosphorylation, reduced channel activity below control (0 mM glucose) levels, consistent with its ability to inhibit metabolism of endogenous nutrients. Although oligomycin substantially lowers intracellular ATP, and thus might be expected to increase intracellular Ca^{2+} , it does not do so under the conditions of our experiments¹⁴; thus its ability to reverse the effect of glucose is not a consequence of Ca^{2+} -dependent inactivation of Ca^{2+} channels⁹. As inhibition of both glycolysis and oxidative metabolism reverses the effect of glucose, a common metabolic product interacting with the Ca^{2+} channel is suggested. Although it is premature to speculate on the nature of this intermediate, we point out that L-type Ca^{2+} channels may be modulated by phosphorylation¹⁵, and that glucose metabolism elevates intracellular ATP levels¹³.

These results may help to explain the increase in electrical activity and insulin secretion produced by increasing glucose concentrations^{5,6}. They also raise the possibility that glucose may modulate dihydropyridine-sensitive Ca^{2+} channels in other tissues, thus providing a mechanism by which metabolism may control cellular processes such as secretion and contraction. □

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Direct modulation of *Aplysia* S-K⁺ channels by a 12-lipoxygenase metabolite of arachidonic acid

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LIPOXYGENASE metabolites of arachidonic acid^{1–3} have recently been shown to modulate the activity of ion channels in nerve^{4–6} and muscle cells^{7,8}. The mechanism of action of these metabolites is, however, unknown. In sensory neurons of *Aplysia*, the S-K⁺ channel is under the dual modulatory control of 5-hydroxytryptamine (5-HT), which decreases the number of active S channels through cyclic AMP-dependent phosphorylation^{9,10}, and the neuropeptide FMRFamide, which increases the probability of S-

channel opening^{11,12} through the 12-lipoxygenase metabolite 12-hydroperoxyeicosatetraenoic acid (12-HPETE)⁴. Here we report that the increase in the probability of S-channel opening with FMRFamide is mimicked by application of 12-HPETE to cell-free membrane patches that lack ATP and GTP. Thus, 12-HPETE can act directly to modulate S-channel activity, independently of protein phosphorylation or dephosphorylation, G-protein activation or cyclic nucleotides.

To test the mode of action of 12-HPETE on S-channel function, we first studied the effects of 12-HPETE on S-channels in cell-free inside-out patches. Application of 12-HPETE to the inside of the membrane produced a dose-dependent reversible increase in S-channel activity (Figs 1 and 4a). In the experiment of Fig. 1, 20 μM 12-HPETE caused an increase in S-channel opening activity to 3.1 times that of control, measured by the

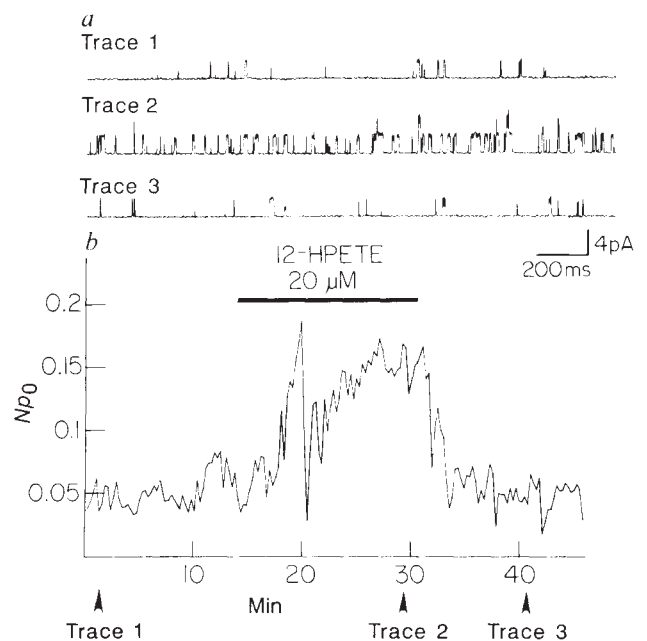


FIG. 1 Effect of 12-HPETE on S-channel activity in inside-out patch. *a*, Single S-channel current records before application of 12-HPETE (trace 1) during application of 20 μM 12-HPETE (trace 2) and after washout of 12-HPETE (trace 3). Patch membrane potential was 0 mV. *b*, Analysis of channel activity measured as Np_0 during entire experiment shown in *a*. 12-HPETE applied during period indicated by bar. Time points at which recordings in *a* were obtained are also indicated. Effect of 12-HPETE is not likely to depend on residual nucleotides or kinases bound to patch because previous results showed that reconstitution of S-channel closure in inside-out patches required combined addition of cAMP-dependent protein kinase and magnesium ATP; kinase alone or ATP alone had no effect¹⁰.

METHODS. Single-channel recordings obtained from pleural ganglion sensory neurons in culture for 1–7 days²². Bath contained 'internal' solution¹⁰ of (in mM): KCl (360), sucrose (246.5), NaCl (10), MgCl_2 (2), EGTA (10), CaCl_2 (1), HEPES buffer (20), pH 7.3. Patch pipettes filled with normal artificial sea water (ASW) solution (in mM) NaCl (460), KCl (10), CaCl_2 (11), MgCl_2 (55), HEPES buffer (10), pH 7.6. S-channels identified by single-channel amplitude (*i*), outwardly rectifying *i*-*V* curve, minimal voltage-dependent gating⁹ and sensitivity to TEA²³. Solutions applied using continuous microperfusion. Speed of microperfusion kept low to maintain patch stability, causing 1–2 min delay between switching solutions and arrival of metabolite at patch. Latencies given in text represent upper limit to true latency. In all experiments, access of bath solutions to membrane assessed using test application of 100 mM TEA. An aliquot of 12(S)-HPETE (Biomol) dissolved in ethanol was dried under argon in the dark immediately before use; internal solution was added to achieve final concentration and mixture sonicated for 15–30 s. Single channel records obtained with a List EPC/7 patch-clamp amplifier, low-pass filtered at 500–1,000 Hz and stored on analogue tape²⁴. Records were digitized by computer (LSI 11/73) at 1–2 kHz. Channel activity measured as Np_0 product from mean number of open channels during 16-s blocks of data²⁵.

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product Np_0 , where N is the number of active channels and p_0 is the probability of opening (see Fig. 1 legend). On average, 20–30 μM 12-HPETE increased S-channel activity to 2.7 ± 1.3 (s.e.m., $n = 5$) times that of control after a variable delay (range, 97–373 s mean, 209 ± 91 s (s.e.m.); $n = 5$).

Although such results clearly show that 12-HPETE can modulate S-channel activity in cell-free patches, the magnitude of the effect is smaller than the four- to fivefold increase in Np_0 produced by FMRFamide in intact cells¹¹. Moreover, compared with the action of 12-HPETE on intact cells, the effect of 12-HPETE on inside-out patches has a slower onset and requires a 10-fold higher concentration (20 μM versus 2 μM)⁴. But, because arachidonic acid metabolites are often released from cells and frequently act on the outside of cell surface membrane receptors^{1–3}, we investigated the action of 12-HPETE on S-channel activity in outside-out patches.

When applied to the outside of the membrane, 12-HPETE produced a larger, more rapid increase in S-channel activity and was effective at submicromolar concentrations (Figs 2 and 4a). Figure 2 shows that 725 nM 12-HPETE produced, with little delay, a 10-fold increase in S-channel opening activity that was

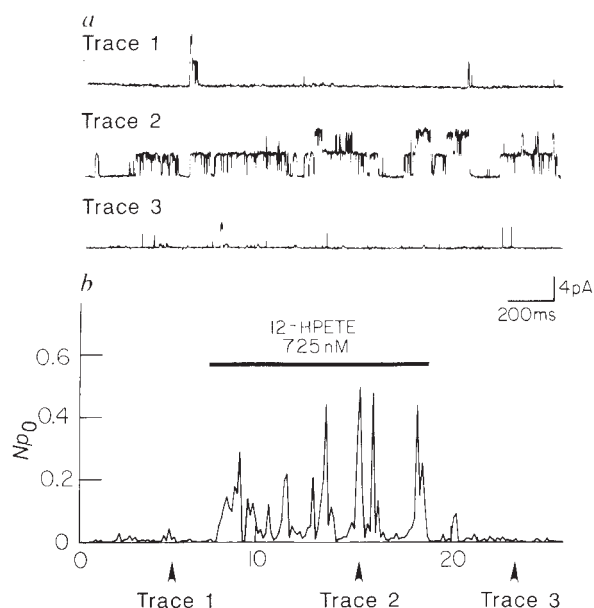


FIG. 2 12-HPETE increases S- K^+ channel activity in outside-out patches of membrane. *a*, Single S-channel currents before (trace 1), during (trace 2) and after (trace 3) washout of 725 nM 12-HPETE. *b*, Plot of Np_0 during experiment. Before application of 12-HPETE, $Np_0 = 0.01$ (averaged over 5-min). During 12-HPETE, Np_0 increased to 0.10 (averaged over 2.5 min). After washout, Np_0 returned to control value of 0.01. In this experiment, 12-HPETE induced bursting behaviour. A similar effect was sometimes observed in response to FMRFamide with intact cells. In other patches, 12-HPETE produced a more sustained increase in channel activity, similar to the effect shown in Fig. 1. Increase in Np_0 is due to an increase in p_0 , because N (estimated from maximal number of channels open simultaneously) does not change, similar to the effects of FMRFamide^{11,12} and arachidonic acid⁴ in intact cells. Timescale in minutes.

METHODS. Bath solution contained normal ASW and pipette contained internal solution (see legend to Fig. 1). 12-HPETE added to normal ASW solution. The difference in effectiveness of 12-HPETE on inside-out and outside-out patches is not due to the different solutions used to apply 12-HPETE (that is 'internal' solution versus normal ASW) because application of 12-HPETE in 'internal' solution to an outside-out patch produced a fivefold increase in Np_0 when at a concentration of 725 nM and a twofold increase in Np_0 at 100 nM, similar to the effects of 12-HPETE applied in ASW. The difference in effectiveness is also probably not due to differences in access of the solutions to the patch because TEA acts equally rapidly to block S-channels when applied to either side of the membrane²³. Finally, these effects are unlikely to result from the presence of nucleotides or kinases attached to the patch, because 5-HT does not close S-channels in outside-out patches.

rapidly reversible. On average, 500–725 nM 12-HPETE produced an 8.0 ± 6.4 -fold increase in Np_0 ($n = 9$) with a mean latency of 40 ± 54 s (range, 0–143 s). At 100 nM, 12-HPETE increased Np_0 3.0-fold in outside-out patches, equal to its effect at 20 μM on inside-out patches (Fig. 4a).

This effect of 12-HPETE was specific in that arachidonic acid (up to 73 μM), the immediate precursor of 12-HPETE, had no significant effect on S-channel activity in inside-out ($n = 4$) or outside-out ($n = 4$) patches (Fig. 3a). This is in contrast to the action of arachidonic acid in increasing S-channel opening activity in intact cells⁴. Similarly, 12-hydroxyeicosatetraenoic acid (12-HETE; 20–30 μM), a metabolite of 12-HPETE that has only a weak effect on intact sensory neurons⁴, had no or only a small effect on S-channel activity in inside-out ($n = 5$) or outside-out ($n = 4$) patches (Fig. 3b). This specific requirement for 12-HPETE is in contrast to the non-specific actions of arachidonate and other fatty acids on channels in lacrimal¹³, cardiac¹⁴ and smooth muscle¹⁵ cells.

The results presented here indicate that 12-HPETE can increase S-channel opening through a direct action on the S-channel or a protein coupled to it in the cell-free patch. Because the bath and pipette solutions contain no ATP or GTP, protein phosphorylation or G-protein activation are not likely to be involved (see also ref. 16). Dephosphorylation is also an improbable mechanism because it cannot explain the reversible increase in S-channel opening activity with 12-HPETE. But because recent biochemical measurements show that FMRFamide does lead to protein dephosphorylation¹⁷, which underlies the reopening by FMRFamide of S-channels closed by 5-HT or cAMP, FMRFamide must have two distinct molecular actions.

The higher efficacy of 12-HPETE at the outside of the mem-

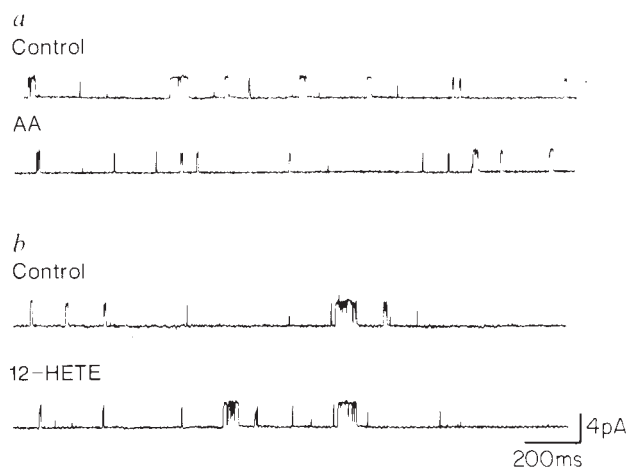


FIG. 3 Effects of arachidonic acid (AA) and 12-HETE on S-channel activity in outside-out patches. *a*, S-channel currents before (control trace) and during (AA trace) application of 20 μM AA. In this experiment, Np_0 during AA application equals 1.0 times that of control. On average, Np_0 in presence of AA (20–30 μM) is 0.74 ± 0.33 times that of control ($n = 4$) for outside-out patches and 1.1 ± 0.15 times that of control for inside-out patches ($n = 4$). *b*, S-channel current before (control trace) and during (12-HETE trace) application of 5 μM 12-HETE. In this experiment, Np_0 during 12-HETE application is 0.6 times that of control. On average, Np_0 in presence of 20–30 μM 12-HETE is 1.1 ± 0.95 times that of control ($n = 4$; range, 0.5–2.5-fold change) for outside-out patches and 2.1 ± 0.8 -times that of control ($n = 5$; range, 1.4–3.6-fold change) for inside-out patches. In other control experiments, the increase in S-channel opening with 12-HPETE is not due to a non-specific effect of the hydroperoxy moiety because 15-hydroperoxyeicosatetraenoic acid (15-HPETE; 20–30 μM) has little or no effect on S-channel activity in inside-out ($n = 1$) or outside-out patches ($n = 2$).

METHODS. AA (Nu-Chek-Prep) was prepared as a 10 mM stock solution in dimethylsulphoxide and kept refrigerated in dark¹⁶. Before use, AA was diluted in ASW (outside-out patches) or internal solution (inside-out patches), vortexed and applied to patch. Final dimethylsulphoxide concentration was 0.2–0.5%. 12(S)-HETE (Biomol) was prepared and applied in the same way as 12-HPETE.

brane compared with that at the inside of the membrane, indicates that the receptor for 12-HPETE could be located on the outside of the membrane¹⁻³. Alternatively, the increased efficacy could result from differential access of the metabolite to the patches (but compare legends of Figs 1 and 2) or the selective presence of a cofactor in the outside-out patch that metabolizes 12-HPETE to a more active compound, such as an epoxy-alcohol^{6,18-20}. A requirement for metabolic conversion was recently proposed by Belardetti *et al.*²⁰, who reported that the efficacy of 12-HPETE on inside-out patches was increased by haematin, an iron-containing compound that catalyses the formation of certain 12-HPETE metabolites¹⁹. An inhibitor of 12-HPETE metabolism, SKF-525A (ref. 21), however, has been shown to only partially block the action of FMRFamide and does not block the action of submicromolar concentrations of 12-HPETE applied to intact cells. Thus we propose that the S-channel contains two modulatory sites for arachidonate metabolites: an external site that responds directly to 12-HPETE and an internal site that is preferentially activated by 12-HPETE metabolites⁶. Finally, the finding that 12-HPETE modulates S-channel activity from either side of the membrane reinforces

the notion⁴ that arachidonate metabolites might be released from the postsynaptic neuron in which they are generated to act as a first messenger to alter the activity of neighbouring neurons. □

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Patch clamp studies of single cell-fusion events mediated by a viral fusion protein

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To enter cells, viruses must fuse their envelope with a host cell membrane. Fusion is mediated by specific, membrane-spanning fusion proteins^{1,2}, of which the influenza virus haemagglutinins (HA) are the best characterized. Several HAs have been sequenced, and the crystal structure of the major part of one HA is known³. The conditions for fusion and some of the rearrangements in the HA that accompany fusion¹⁻³ are well understood, but it remains unclear how HA causes bilayers to fuse. We have observed, in real time, unitary cell-fusion events caused by HA. Fibroblasts expressing HA were induced to fuse with red blood cells by a rapid drop in pH⁴. Fusion was monitored by fluorescence microscopy, and by measuring the membrane conductance and capacitance of the fibroblast. The earliest event observed was the sudden opening of an aqueous pore connecting the cytoplasm of the fusing cells. Initially, the pore conductance often fluctuated between zero and ~600 pS, as if the pore were opening and closing repeatedly. Later, it increased over tens of seconds, as if the pore dilated. We suggest that, as in exocytosis⁵, HA-mediated membrane fusion begins with the formation of a narrow pore. Based on the conductance, we estimate the initial diameter of the pore to be no more than twice that of a gap junction channel⁶.

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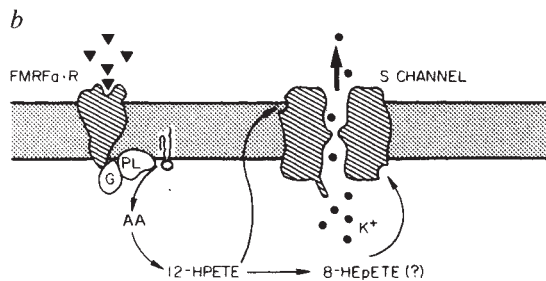
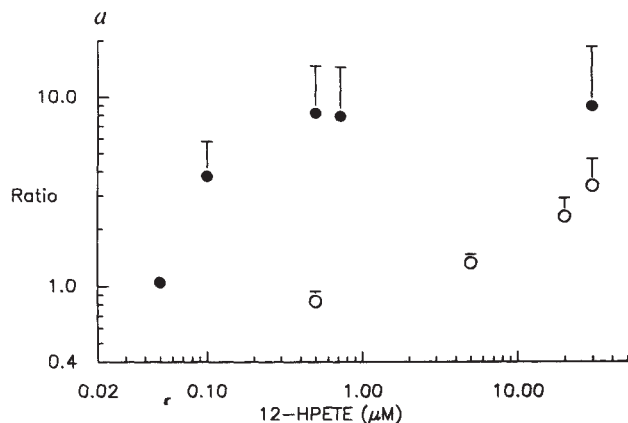


FIG. 4 12-HPETE action on inside and outside of membrane. *a*, Dose-response curves for 12-HPETE on inside-out (○) and outside-out (●) patches. Note log-log scale. Error bars, s.e.m. Ratio gives mean change in Np_o in presence of 12-HPETE compared with average of two control values for Np_o , one obtained before 12-HPETE and one obtained after washout. Values for Np_o measured from 2.5-5-min stretches of data. The value for Np_o in the presence of 12-HPETE was measured using a similar stretch of averaged data, obtained around the time of the maximal increase in Np_o . *b*, Model for dual modulation of S-channel by 12-HPETE and downstream metabolites. Binding of FMRFamide (FMRFa) to receptor (R) activates a pertussis-toxin sensitive G protein¹⁶ which activates a phospholipase (PL), releasing AA. 12-HPETE produced by 12-lipoxygenase can directly interact with a site near outer surface of membrane. In addition, further metabolism of 12-HPETE may produce compounds, such as 8-hydroxy-11,12-epoxy-eicosatrienoic acid (8-HEpETE)⁶, that act at inner surface of membrane. Exogenous 12-HPETE can primarily act at external site, especially at low concentrations, thus providing an explanation for why blockade of 12-HPETE metabolism has little effect on response to 12-HPETE²⁰.

Fibroblasts of the cell line NIH3T3-HA-b2 (refs 4, 7) express influenza virus haemagglutinin trimers at high density on their plasma membrane. They bind erythrocytes (RBCs) at sialic acid binding sites on the HA. Once HA has been cleaved to its fusion-competent form by mild trypsinization, it can be triggered by acidification to cause fusion. Figure 1a shows a fusion-competent fibroblast, decorated with three RBCs (arrows) that had been previously loaded with the fluorescent dye, lucifer yellow (Fig. 1b). After acidification from pH 7.4 to pH 4.8, the fibroblast became fluorescent and the RBCs dimmed (Fig. 1d). Evidently the dye had diffused from the RBCs into the fibroblast, indicating cytoplasmic continuity, or fusion, between the two cell types. The RBCs remained attached to the fibroblast (Fig. 1c). No transfer of dye was seen unless the medium was acidified, and then only if the fibroblasts had previously been trypsinized. Hence fusion took place only under conditions known to activate the HA, confirming earlier findings⁴.

To study the time course of fusion, we measured the fibroblast's membrane capacitance, C , reasoning that the additional cell surface area of any RBC fusing to the fibroblast should

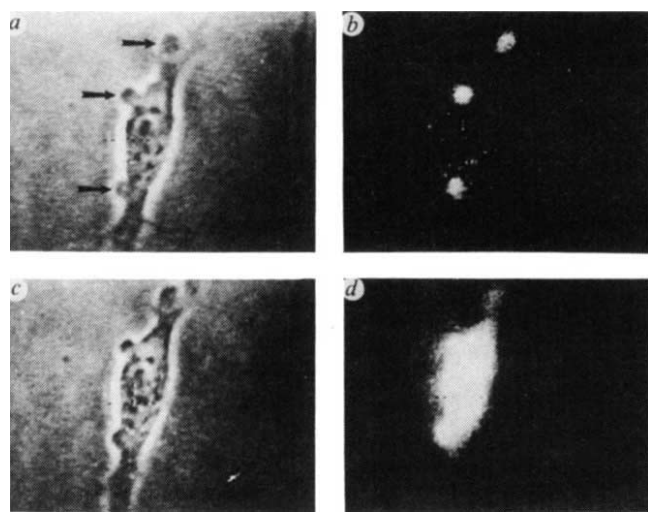


FIG. 1 Phase (a, c) and fluorescence micrographs (b, d) of a fusogenic NIH3T3-HA-b2 fibroblast with three resealed erythrocyte ghosts attached (arrows in a). Ghosts were prepared as described⁷ and loaded with lucifer yellow (M_r 457); they fluoresce brightly (b). a, b, Micrographs were taken in Tyrode solution (140 mM NaCl, 2.5 mM KCl, 5 mM MgCl₂, 2 mM CaCl₂, 2 mM Na-HEPES, pH 7.4) and in c, d, 5 min after starting a stream of acidic Tyrode (containing 20 mM sodium succinate at pH 4.8 instead of HEPES) from a glass micropipette (tip diameter 5 μ m) placed to within 100 μ m of the cell. In d, fluorescence has migrated from the RBCs into the fibroblast, indicating cytoplasmic continuity, that is, cell fusion at 28 °C. In other experiments, cells were incubated at 37 °C in acidic Tyrode for 5 min and then observed for fluorescence. Of all fibroblasts carrying a single RBC, 72 $\pm$ 2% became fluorescent (6 experiments with >100 fibroblasts observed in each). When other dyes were used (fluorescein-conjugated dextran, M_r 10,000, or the carboxyfluorescein, BCECF, M_r 520) the chance of fluorescence transfer was less (31 $\pm$ 8% and 26 $\pm$ 11%, respectively). Scale bar, 20 μ m.

METHODS. NIH3T3-HA-b2 fibroblasts were maintained in a growth medium as described⁴ and grown to 30–60% confluency on glass coverslips. To cleave the HA into its fusion-competent form, cells were washed in PBS, incubated in serum-free growth medium containing trypsin (10 μ g ml⁻¹) for 5 min at 22 °C, then washed with growth medium and returned to the incubator. For experiments, individual coverslips covered with fibroblasts were removed 15–120 min later, and decorated with fresh human RBCs as follows. RBCs were washed in PBS, pelleted and resuspended at 10,000–15,000-fold dilution. A 2-mm layer of this suspension was dripped onto the coverslip, and the RBCs contained in it were centrifuged onto the fibroblasts at 380g for 3 min. RBCs that did not bind to fibroblasts were washed off. Cells were viewed with a $\times$ 40, 0.65 numerical aperture objective.

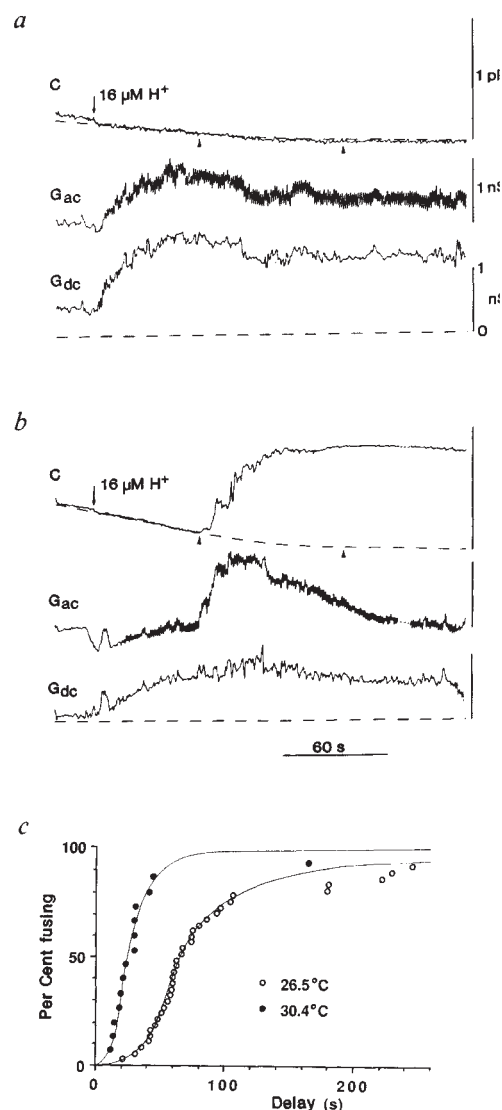


FIG. 2 a, b, Capacitance (C) and conductance (G_{ac} , G_{dc}) changes in trypsinized NIH3T3-HA-b2 fibroblasts while the pH of the solution was stepped from 7.4 to 4.8 (arrows in a, b), as in Fig. 1. G_{ac} is measured under sinusoidally alternating voltage, G_{dc} under steps to fixed voltage levels. a, Without RBC; b, with one RBC attached to the fibroblast. Dashed lines in the two C traces, see legend to Fig. 3. Calibrations as in a. c, Cumulative distribution of fusion delays. Cells from experiments as in b and Fig. 4 were ranked according to the delay between acidification and the first sign of fusion-related changes in C and G_{ac} . The rank was plotted on the ordinate and the ordinate scaled to 100%. Temperatures $\pm$ s.d. were 26.5 $\pm$ 1.2 °C (n = 37) and 30.4 $\pm$ 1.2 °C (n = 15). The plots include one (30.4 °C) and three (26.5 °C) cases where an attached RBC failed to fuse. Lines fitted by eye.

METHODS. We voltage-clamped fibroblasts in the 'whole-cell' patch clamp mode²⁰. Glass micropipettes (filled with 155 mM potassium glutamate, 10 mM Na-HEPES, 5 mM MgCl₂, 5 mM K-BAPTA, pH 7.4) had resistances of 2–4 M Ω in Tyrode and established 2.5–6 M Ω connections with the cytoplasm. We used small solitary fibroblasts (capacitances 14.0 $\pm$ 0.4 pF; conductance 153 $\pm$ 25 pS; n = 37; 25–28 °C), with either no RBCs (a) or single RBCs (b) attached in the perinuclear region. Once per second, the fibroblast's membrane potential was stepped for 0.5 s from -10 mV to 0 mV or from 0 to 10 mV. The resulting current steps were used to calculate the d.c. conductance (G_{dc}). In addition, a 320 Hz sinusoid (30 mV peak-to-peak) was superimposed on the voltage steps. The resulting sinusoidal current was separated with a lock-in amplifier²¹ into its real (in phase with the voltage) and imaginary (90° out of phase) components. This yielded the real (G_{ac}) and imaginary (C) components of the electrical admittance between inside and outside of the fibroblast. The phase setting on the lock-in amplifier was adjusted to compensate for the electrical delays resulting from the recording equipment (List EPC-7) and from the resistance of the micropipette. Traces connect data points that are time averages over 0.5-s intervals.

cause an increase in C . In Fig. 2, acidic solution was applied to an HA-expressing fibroblast. Figure 2a was recorded from a fibroblast that carried no RBCs and hence lacked a fusion partner. As expected, C did not change significantly. The only change seen was a slow decline ($\sim 5\%$) that probably occurred because our method of measuring C slightly changes the composition of the cytosol, and hence may stimulate endocytosis. We also measured the membrane conductance of the fibroblast, both with alternating current (G_{ac}) and with constant-voltage pulses (G_{dc}). G_{dc} reflects the conductance due to ion channels in the plasma membrane, whereas G_{ac} represents the sum of G_{dc} and the conductance of any connection between the fibroblast and a fusing RBC. Because of the RBC membrane, such a connection would not contribute to G_{dc} (Fig. 3b, insert). In Fig. 2a, it can be seen that acidification causes a conductance increase visible on both the G_{ac} and G_{dc} traces (404 ± 90 pS; $n = 27$; 27°C ; values are given $\pm$ standard error unless stated otherwise). The effect was reversible (data not shown). Protons probably led to the opening of ion channels in the fibroblast membrane. Neither the slight decline in C , nor the conductance increase are due to the HA as both were also observed in NIH3T3 fibroblasts that do not express HA ($n = 2$).

Figure 2b shows an identical experiment on a fibroblast decorated with one fresh RBC. As in Fig. 2a, protons caused G_{dc} to increase; the G_{ac} trace showed the superimposition of an additional, transient increase. Most importantly, C increased markedly after a delay (first arrowhead). C fluctuated as it increased, but later stabilized at a new, higher level. The C increase must have been due to fusion of the RBC to the fibroblast, because (1) it was never observed without an attached RBC, and (2) in whole-cell patch clamp of RBCs, the capacitance of single RBCs was 910 ± 20 fF ($n = 13$), identical to the final value of the C increase observed in Fig. 2b (mean 930 ± 30 fF; $n = 27$). Moreover, the fusion occurred only under conditions known to activate HA. It was never observed without prior trypsin treatment, and even in trypsinized fibroblasts, it never occurred without acidification. We conclude that the C increase represents the HA-mediated fusion of a single RBC to a fibroblast. As the G_{ac} conductance transient always accompanied the increase in C , it too must be related to fusion. Out of 52 such experiments, an attached RBC failed to fuse in only four cases, hence the probability of fusion is 92%.

The delay between acidification and the first sign of fusion-related C and G_{ac} changes (median 63 s at 26.5°C , $n = 34$; and 27 s at 30.4°C , $n = 14$) is analysed in Fig. 2c, which shows the per cent of cells that fuse within the time shown on the abscissa and traces the time course of the early steps in the fusion reaction. The relationship is steeply temperature-dependent, consistent with earlier work⁸. The sigmoidal time course indicates a multi-step reaction, and may reflect a need for several HA trimers to aggregate before fusion.

Figure 3a replots the two fusion-related signals of Fig. 2b. We have subtracted from the C trace the slow decline in C observed in the absence of fusion (simulated by the dashed line in Fig. 2b). Note that C continues to increase for 40–50 s, and that, while C is growing, the G_{ac} transient goes through a maximum. Both features are expected if an electrical connection between fibroblast and RBC forms and then increases gradually. This is illustrated in Fig. 3b (insert), which depicts an RBC (with capacitance C_{RBC}) connected to a fibroblast by a pore of conductance g . While the pore is narrow and g small, the voltage sinusoid with which we measure C invades the RBC only incompletely, and hence C_{RBC} will not be measured fully. Only as the pore widens and g increases will all of C_{RBC} be gradually revealed. The G_{ac} transient must also reflect changes in pore conductance. Because this conductance change is observed only with a.c. and not with d.c., it cannot result from ion channels in the fibroblast plasma membrane and must instead arise in a conductance (g) in series with a capacitor (C_{RBC}). To understand why a widening pore causes a transient increase in G_{ac} ,

consider the series combination of g and C_{RBC} as a voltage divider. While g is small, the sinusoidal voltage drops mainly across the resistance of the pore, so that the RBC appears electrically as an added conductance that increases as the pore widens. This continues until C has reached half its final value

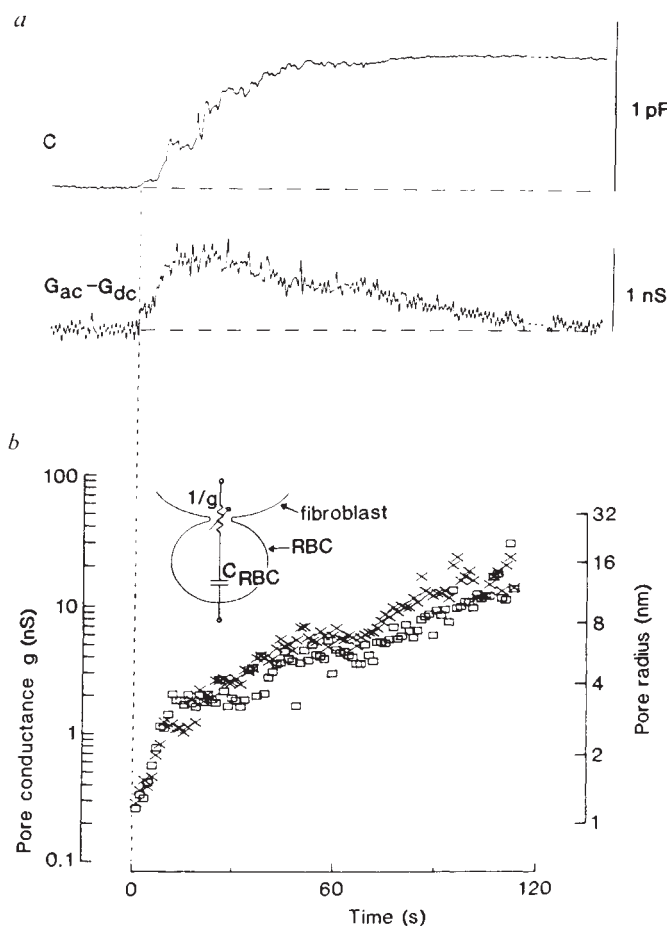


FIG. 3 a, Fusion-related C and G_{ac} changes. C trace is from Fig. 2b after subtracting a reference trace (C_{ref} ; dashed line in Fig. 2b) that simulates the decline of C observed in the absence of fusion. To obtain C_{ref} , the C trace in Fig. 2b is divided into three sections. In the first (from application of H^+ to position indicated by first arrowhead), C_{ref} is a straight line fitted to the C trace. Between arrowheads, C_{ref} is a parabola calculated by changing the slope at a constant rate until it equals that of a straight asymptote (not shown) fitted to the final section of the C trace. In the last section, C_{ref} continues as a straight line parallel to the C trace. This procedure was also applied to the C trace in Fig. 2a (dashed line) and is seen to simulate it well. $G_{ac} - G_{dc}$ represents the difference between G_{ac} and G_{dc} traces in Fig. 2b. b, Inset: Schematic drawing and electrical representation of a fibroblast joined to a RBC by a fusion pore. Below, pore conductance (g) as a function of time⁵. Crosses calculated from the C trace by

$$g = \frac{2\pi f C_{RBC}}{\sqrt{C_{RBC}/C - 1}} \quad (1)$$

where f is the frequency of the sinusoid (320 Hz) and C_{RBC} is the final C displacement in a. Boxes calculated from $G_{ac} - G_{dc}$ by

$$g = \frac{2G}{1 + n\sqrt{1 + (G/\pi f C_{RBC})^2}} \quad (2)$$

where $G = G_{ac} - G_{dc}$ and $n = 1$ while $C < C_{RBC}/2$, and -1 otherwise. Each symbol shows the time average over 1 s. Right-hand ordinate is calculated from g (ref. 5), assuming g to be due to a single dilating pore of resistivity $100 \Omega\text{cm}$ (similar to Tyrode solution) and length 15 nm (about the length of a gap junction channel⁶ or a HA trimer³).

and half the voltage drops across C . Then the series combination of the two elements will appear more and more like a capacitor and less like a conductor, hence G_{ac} declines again.

The two traces in Fig. 3a can each be used independently to calculate the pore conductance g , as a function of time (Fig. 3b). The agreement between the two calculations supports the idea that the G_{ac} transient and the gradual increase in C both reflect a pore conductance that continues to grow over >2 min. After the first sign of fusion, it takes 44 ± 9 s (26.5°C) for g to reach a value of 2.5 nS. This is at least 1,000 times slower than during exocytosis in mast cells⁵. The right-hand ordinate converts g into pore radius. Clearly, the pore is narrow at first and

dilates only slowly to a size that would accommodate, for example, the nucleocapsid of an influenza virus. The pore's reluctance to dilate may be related to cytoskeletal constraints on the RBC membrane.

Ion channels⁹ and exocytic fusion pores^{5,10,11} can rapidly and repeatedly 'flicker' open and shut. As heavily filtered recordings (for example, in Fig. 3c) may underestimate the conductance of a flickering pore, we have examined the early fusion events with a method that can resolve changes occurring within as little as 6–9 ms. In Fig. 4 and in all of four similar experiments, fusion started with a small increase in C and a simultaneous, sudden increase in G_{ac} . G_{dc} does not change at that time, hence the sudden G_{ac} change cannot result from the opening of ion channels in the fibroblast, and must reflect the opening of the fusion pore. The lack of a d.c. pathway early in fusion (<100 pS during the first 20 s in Fig. 4a) indicates that fusion was tight^{1,12}. Figure 4b shows sections of the traces in Fig. 4a in more detail: G_{ac} increases abruptly to ~ 600 pS and then remains there for nearly a second, with only small interruptions. In another fusion (Fig. 4c), the pore showed signs of opening and closing repeatedly (flicker), suggestive of reversible intermediate steps in fusion. By equations (1) and (2), the initial C and G_{ac} deflections (dashed lines in Fig. 4) indicate a pore conductance of 623 ± 69 pS ($n = 5$). On the right-hand ordinate of Fig. 3, this would translate to a pore diameter of ~ 4 nm. We cannot exclude still smaller precursors with lifetimes of milliseconds.

We report time-resolved recordings of single membrane-fusion events mediated by a viral fusion protein. The first sign of fusion is the formation of a narrow aqueous pore connecting the fusing cells. The opening of the pore occurs at least as rapidly (27 s after acidification at 30.4°C) as the first evidence of lipid mixing in fluorescence-dequenching measurements¹³ on RBCs and HA-bearing fibroblasts (after 54 s at 30°C ; refs 8 and 14). Hence the fusion pore may be the earliest fusion event recorded to date. Initially it must have molecular dimensions, because its conductance (600 pS or less) falls in the range observed with proteinaceous pores such as gap junction channels^{15,16} (80–240 pS) and pores formed by complement or by the cytolytic protein of cytotoxic T lymphocytes¹⁷ (1–2 nS). Intramembrane particles possibly representing HA-mediated fusion pores have been observed in freeze-fracture¹⁸.

The fusion events observed here may be compared with exocytosis in mast cells. As in HA-mediated fusion, exocytic fusion starts with the opening of a narrow pore (200–300 pS initial conductance^{5,19}) and has reversible intermediates as evidenced by 'flicker'. In both types of fusion, the initial pore could share similarities with ion channels. $\square$

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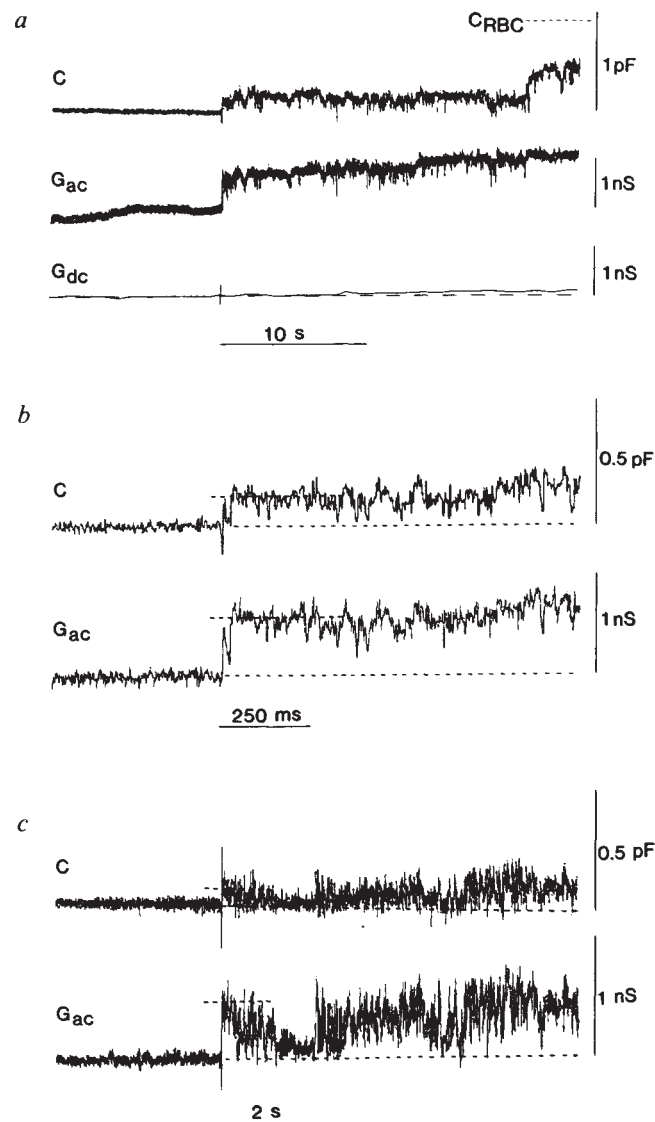


FIG. 4 a, C and G_{ac} were measured during each sinusoidal cycle by a high-speed lock-in amplifier using phase-locked integration⁵. The traces connect discrete points sampled once every 3.13 ms, hence they resolve events occurring at times down to 6–9 ms. Voltage steps were omitted, so G_{dc} was not measured. Acidification occurred 30 s before the first signs of fusion, that is, the jumps in C and G_{ac} at 31°C . The dashed line indicates the average C_{RBC} , or 0.9 pF. G_{dc} is the average of 21 traces as in Fig. 2b, aligned in time so that the first G_{ac} change indicative of fusion occurred at the vertical line. b, Sections of the corresponding traces in a shown at higher speed. c, From another cell, but recorded as in a. Time from acidification to fusion was 13 s at 29°C . Dashed lines from equations (1) for C and (2) for G_{ac} , assuming $C_{RBC} = 0.9$ pF and $g = 650$ pS (b) or 512 pS (c).

Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen

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THE crucial role of the thymus in immunological tolerance¹⁻⁵ has been demonstrated by establishing that T cells are positively selected to express a specificity for self major histocompatibility complex (MHC)⁶⁻⁸, and that those T cells bearing receptors potentially reactive to self antigen fragments, presumably presented by thymic MHC, are selected against⁹⁻¹¹. The precise mechanism by which tolerance is induced and the stage of T-cell development at which it occurs are not known. We have now studied T-cell tolerance in transgenic mice expressing a T-cell receptor with double specificities for lymphocytic choriomeningitis virus (LCMV)-H-2D^b and for the mixed-lymphocyte stimulatory (Mls^a) antigen. We report that $\alpha\beta$ TCR transgenic mice tolerant to LCMV have drastically reduced numbers of CD4⁺CD8⁺ thymocytes and of peripheral T cells carrying the CD8 antigen. By contrast, tolerance to Mls^a antigen in the same $\alpha\beta$ TCR transgenic Mls^a mice leads to deletion of only mature thymocytes and peripheral T cells and does not affect CD4⁺CD8⁺ thymocytes. Thus the same transgenic TCR-expressing T cells may be tolerized at different stages of their maturation and at different locations in the thymus depending on the antigen involved.

Transgenic mice expressing the P14-TCR specific for LCMV-H-2D^b ($V\alpha_2J\alpha_{TA31}/V\beta_{8.1}D\beta J\beta_{2.4}$) (refs 12, 13) offer the unique

TABLE 1 Peripheral T-cell subsets in transgenic mice

Mice	T-cell population	Percentage of lymph node cells	Population expressing $V\beta_8$ (%)	Absolute numbers per spleen ($\times 10^6$)
$\alpha\beta$ -transgenic H-2 ^b , Mls ^b	CD3	55 $\pm$ 5	92 $\pm$ 3	14.9 $\pm$ 3.0
	CD4	9 $\pm$ 3	62 $\pm$ 5	3.0 $\pm$ 0.5
	CD8	48 $\pm$ 11	94 $\pm$ 2	10.1 $\pm$ 2.3
$\alpha\beta$ -transgenic H-2 ^{b,d} , Mls ^a	CD3	26 $\pm$ 3	62 $\pm$ 8	3.3 $\pm$ 1.5
	CD4	9 $\pm$ 2	25 $\pm$ 3	1.2 $\pm$ 0.6
	CD8	16 $\pm$ 4	83 $\pm$ 5	1.5 $\pm$ 0.8
$\alpha\beta$ -transgenic LCMV-carrier H-2 ^b , Mls ^b	CD3	36 $\pm$ 4	67 $\pm$ 9	4.9 $\pm$ 1.2
	CD4	10 $\pm$ 3	23 $\pm$ 4	1.4 $\pm$ 0.4
	CD8	4 $\pm$ 1	33 $\pm$ 3	0.5 $\pm$ 0.1

Lymph-node cells of the indicated mice were stained with KJ16 monoclonal antibody (MAb)²¹ (anti- $V\beta_{8.1+8.2}$) and (FITC)-conjugated goat anti-rat IgG (TAGO, Burlingame, California). The staining was followed either by biotinylated anti-CD8 monoclonal antibody (Becton-Dickinson) and phycoerythrin (PE)-conjugated avidin (TAGO) or by PE-conjugated anti-CD4 monoclonal antibody (Becton-Dickinson). For the CD3/ $V\beta_8$ double staining, cells were first stained with F23.1 (anti- $V\beta_8$ monoclonal antibody²²) and FITC-conjugated anti-mouse Ig2a (Southern Biotechnology, Birmingham, Alabama) followed by KT3 mAb²³ (anti-CD3) and PE-conjugated goat anti-rat IgG (Southern Biotechnology). The average percentages (after subtraction with the fluorescein conjugate alone (<2%) of three mice per group are shown. Absolute values per spleen were calculated on the basis of the total number of spleen cells and cytofluorometric analysis of the cells stained with anti-CD3, CD4 and CD8 monoclonal antibodies. Red blood cells were removed before cytofluorometric analysis with FACS lysing solution (Becton-Dickinson). The data represents the analysis of three mice (6-12 weeks of age) per group.

possibility to examine T-cell tolerance to two independent antigens with the same transgenic mouse line. First, T-cell tolerance to LCMV has been studied in transgenic mice carrying the non-cytopathic LCM virus after neonatal infection¹⁴. Second, the transgenic TCR use the β -chain variable gene segment $V\beta_{8.1}$ that reacts preferentially with Mls^a antigen: in Mls^a mice, therefore, such $V\beta_{8.1}$ T cells are clonally eliminated in the thymus¹⁰.

In a 3-day *in vitro* stimulation, spleen cells from uninfected transgenic Mls^b and Mls^a mice, but not from transgenic LCMV-carrier mice (Mls^b) or from normal unprimed mice, showed specific primary immune responses by proliferation and cell-mediated lysis of LCMV-infected target cells (Fig. 1). The response of transgenic Mls^a spleen cells to LCMV was three times less than that of transgenic Mls^b spleen cells; this decrease was probably due to the reduced number of CD8⁺ T cells in the spleen of transgenic Mls^a mice (Table 1), because comparable frequencies of LCMV-specific T-cell precursors among CD8⁺ T cells were found in transgenic Mls^a and Mls^b mice. The frequency of LCMV-specific cytolytic T-cell precursor cells among CD8⁺ T cells was determined, by limiting dilution analysis, to be 1/1.8-4.2 in Mls^b $\alpha\beta$ -transgenic mice, 1/2.4-3.9 in Mls^a $\alpha\beta$ -transgenic mice, and below 1/10⁴ in normal unprimed mice¹⁵.

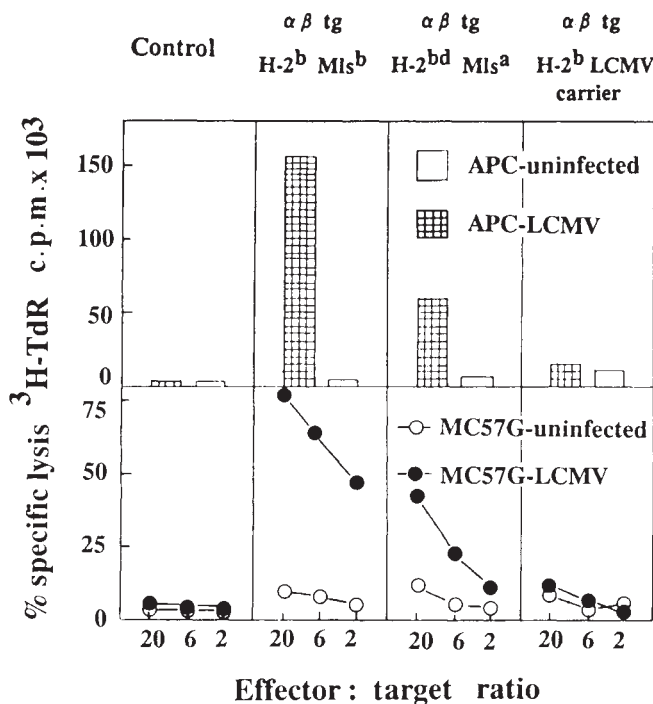


FIG. 1 Spleen cells from $\alpha\beta$ transgenic Mls^b and Mls^a mice generate a primary anti-LCMV cytotoxic T-cell response *in vitro* within three days.

METHODS. The $\alpha\beta$ transgenic mice were generated by co-injection of the P14 TCR α - and β -chain gene constructs²⁵ into (C57BL/6 $\times$ DBA/2) F₂-generation fertilized eggs. The male founder 327 bearing 10-20 copies of both α - and β -transgenes integrated at the same chromosome was backcrossed to C57BL/6 (H-2^b, Mls^b) mice. $\alpha\beta$ -transgenic Mls^a mice were obtained by mating the H-2^b, Mls^b transgenic line with DBA/2 (H-2^d, Mls^a) mice, and $\alpha\beta$ -transgenic LCMV-carrier mice were generated by neonatal LCMV infection (<24 h after birth; 5×10^6 plaque-forming units (PFU) LCMV-WE strain per newborn) of transgenic H-2^b, Mls^b offspring. To examine the T-cell activities of these mice, spleen cells (4×10^6 ml⁻¹) were cultured with irradiated (3,000 rad) LCMV-infected and uninfected peritoneal macrophages (4×10^5 ml⁻¹) from C57BL/6 mice. Cell proliferation was determined after 2 days by ³H-labelled thymidine uptake ³H-TdR (upper panel). The lytic activities (lower panel) of the LCMV-stimulated cultures were determined after 3 days against LCMV-infected and uninfected MC57G (H-2^b) target cells in a 4-5 h ⁵¹Cr-release assay, as described previously¹². The data show a representative experiment for the three types of $\alpha\beta$ -transgenic mice.

(Table 2). These findings indicate that the clonal deletion of transgenic receptor-bearing T cells due to Mls^a was incomplete.

Thymus size and total thymocyte numbers of $\alpha\beta$ -transgenic Mls^a and Mls^b mice did not differ significantly from those of their transgene-negative litter-mates. In marked contrast, thymus size and total thymocyte numbers of transgenic LCMV-carrier mice were drastically reduced (1–10% those of negative litter-mates). A strong skewing towards the carrying of CD8 antigen was observed for thymocytes and peripheral T cells of transgenic H-2^b Mls^b mice (Fig. 2b and Table 2) but not for those of transgenic H-2^d Mls^b mice (data not shown). This reflects the origin of the transgenic TCR from a H-2^b-restricted CD8⁺ T-cell clone, and in addition provides evidence for the positive selection of T cells by thymic MHC in the absence of the nominal antigen. These results confirm observations in H-Y antigen-specific and alloreactive TCR transgenic mice^{7,8}.

Double staining with CD4- and CD8-specific antibodies revealed high numbers of TCRV β ⁺ CD4⁺CD8⁺ thymocytes (~80%) in transgenic Mls^a mice (Fig. 2c, g). The mature single CD8⁺ thymocyte subset, however, was clearly reduced compared with that of transgenic Mls^b mice (2.3% versus 18.6%). Correspondingly, the number of mature thymocytes expressing a high TCR density were decreased in Mls^a mice. Transgenic TCR V β _{8.1}⁺ CD8⁺ peripheral T cells did not exhibit an Mls^a reactivity *in vitro* (data not shown). Only 31% of the few remaining thymocytes of $\alpha\beta$ -transgenic LCMV-carrier mice were double positive for CD4CD8, and the percentage of thymocytes expressing V β ₈ was drastically reduced (Fig. 2d, h). Single CD4⁺ (14.8%) and CD8⁺ (9.1%) thymocytes carried the CD3 antigen but were mostly V β ₈⁺, indicating that the transgenic β -chain gene was not expressed on these cells (Fig. 2o, p). The

CD4⁺CD8⁺ thymocytes of transgenic LCMV-carrier mice expressed high levels of CD3 antigen (Fig. 2m) but only low levels of TCR V β ₈ and $\alpha\beta$ determinants (Fig. 2o, p). These cells probably belong to the thymic $\gamma\delta$ -compartment which was not affected by tolerance induction, and the relative but not the absolute frequency of these cells increased through the massive deletion of $\alpha\beta$ CD4⁺CD8⁺ thymocytes.

The drastically reduced number of CD4⁺CD8⁺ thymocytes and of transgenic receptor-bearing CD8⁺ peripheral T cells (Table 2) is direct evidence of tolerance induction by clonal deletion in LCMV-carrier mice. Clonal deletion seems to occur in the thymic cortex, where LCMV has been shown to be present in neonatally infected mice¹⁶. The fact that ~60% of the V β ₈⁺ T cells in transgenic carrier mice did not express detectable CD4 or CD8 molecules indicates that besides deletion, down-regulation of CD8 could be a different pathway to achieve nonreactivity of transgenic TCR-bearing T cells¹⁷. In general our results parallel the observations made with H-Y antigen-specific¹⁷ and alloreactive (H-2L^d)⁸ transgenic TCR mice. The study using the LCMV model extends these observations by demonstrating that besides self antigens (MHC, H-Y, Mls), foreign antigens (for example, LCMV) are also able to induce deletion of reactive T cells when introduced at an early stage of T-cell development.

Because of the double specificity of the transgenic TCR (LCMV-H-2^b, Mls^a), tolerance to Mls^a could be examined with the same transgenic mouse line. In contrast to the $\alpha\beta$ -transgenic LCMV-carrier mouse, thymocyte numbers and CD4⁺CD8⁺ thymocytes were not affected in transgenic Mls^a mice; the single CD8⁺ thymocyte subset and peripheral CD8⁺ T cells, however, were clearly reduced compared with those of transgenic Mls^b mice (Table 2).

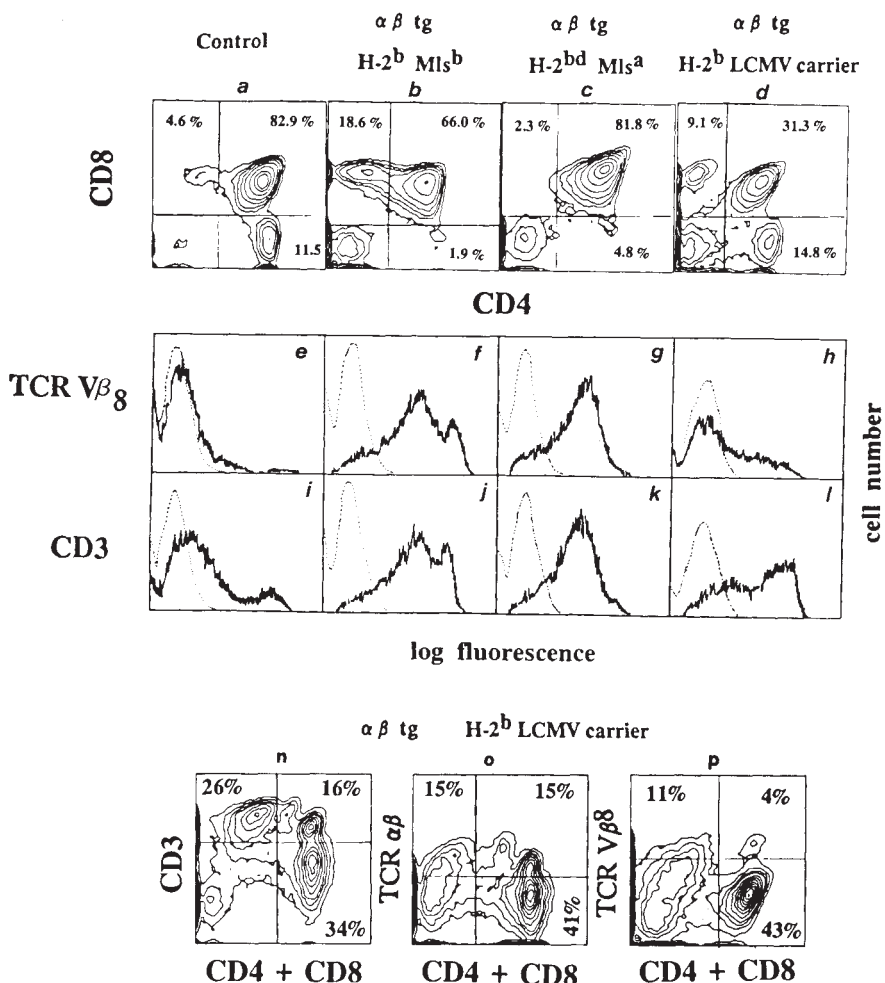


FIG. 2 Expression of CD3, CD4, CD8, $\alpha\beta$ TCR and V β ₈ TCR molecules on thymocytes from nontransgenic control, P14 $\alpha\beta$ TCR transgenic Mls^b, Mls^a, and LCMV-carrier (H-2^b, Mls^b) mice. In the first panel (Fig. 2a-d) thymocytes were stained with PE-conjugated anti-CD4 and FITC-labelled anti-CD8 monoclonal antibody. The second panel (Fig. 2e-h) shows single staining profiles (solid lines) with KJ16 (anti-V β _{8.1+8.2}) and KT3 (anti-CD3) monoclonal antibodies followed by FITC-conjugated goat anti-rat IgG. Dotted lines indicate staining with the fluorescent conjugate alone. The third panel (Fig. 2i-l) shows two-colour flow-cytometric analysis of CD4CD8/TCR expression on thymocytes from $\alpha\beta$ -transgenic LCMV-carriers mice. The cells were incubated first with KT3, KJ16 and H57-597 (anti- $\alpha\beta$ TCR) monoclonal antibodies and goat FITC-conjugated anti-rat/hamster IgG followed by PE-conjugated anti-CD4 and biotinylated anti-CD8 monoclonal antibody and finally PE-streptavidin.

METHODS. Single-cell suspension of thymocytes were stained in PBS with 2% FCS and 0.2% sodium azide with the various antibodies. Incubations were for 30 min at 4 °C (except for KJ16 and H57-597, which was at 37 °C). First-step reagents used were monoclonal antibodies anti CD4-PE, FITC-conjugated anti-CD8, biotinylated anti-CD8 (Becton-Dickson); KT3²³, KJ16²¹, H57-597²⁶. Second-step reagents used were FITC-conjugated goat anti-rat IgG (TAGO), FITC-conjugated goat anti-hamster IgG FITC (Jackson Immunoresearch) and PE-streptavidin (Becton-Dickson). Viable cells (50,000) per sample were analysed by flow cytometry on a Epics Profile Analyzer with logarithmic scales. Viable cells were gated by a combination of narrow-angle forward light-scatter and perpendicular scatter. Tg, Transgenic.

TABLE 2 Frequency of LCMV-specific cytotoxic T-cell precursors among CD8⁺ T cells of TCR $\alpha\beta$ transgenic mice

Mice	Reciprocal frequencies
C57BL/6	>10 ⁴ (ref. 15)
Uninfected	
C57BL/6 acutely infected with LCMV, day 8	3.0–4.1
$\alpha\beta$ -transgenic H-2 ^b , Mls ^b	1.8–4.1
Uninfected	
$\alpha\beta$ -transgenic H-2 ^b d, Mls ^a	2.4–3.9
Uninfected	
$\alpha\beta$ -transgenic H-2 ^b , Mls ^b	>10 ⁴
Neonatally infected LCMV-carrier	

Limiting numbers of responder spleen cells were cultured in 96-well round-bottom plates with irradiated (3,000 rad) LCMV-infected peritoneal macrophages (10³ cells per well) and with irradiated spleen filler cells from LCMV-infected mice (10⁵ cells per well) in the presence of 25% (v/v) supernatant from concanavalin A-stimulated rat spleen cells. The precise number of CD8⁺ T cells was determined by staining of an aliquot of the used responder spleen population with CD8-specific monoclonal antibody and cytofluorometric analysis. After 7 days, the cytolytic activities of the microcultures were tested on LCMV-infected and noninfected MC57G target cells in a ⁵¹Cr-release assay¹². Cultures were scored as positives when LCMV-specific lysis was >15%. Frequencies were calculated according to Taswell²⁴.

Thus tolerance induction involving the same TCR can vary with the antigen involved. It could be that the time-dependent appearance or the localization of the two antigens (LCMV, Mls^a), or both of these factors, are different in the thymus. In LCMV-carrier mice, LCMV antigens have been detected very early throughout the thymus¹⁶, so thymocytes could meet LCMV antigen and MHC class I molecules in the thymic cortex and medulla. The distribution of the Mls^a antigen is not known. But because Mls^a antigen is recognized by T cells in the context of MHC class II molecules which are not detected in the outer cortex¹⁸, deletion of most double CD4⁺CD8⁺ cells would not occur in $\alpha\beta$ -transgenic Mls^a mice. Alternatively, the affinity of the transgenic receptor for LCMV-H-2D^b could differ from that for Mls^a. Double positive thymocytes having a high-affinity receptor for the appropriate antigen (LCMV-H-2D^b in our model) are therefore deleted at an early state of development. Thymocytes expressing a low-affinity receptor to Mls^a would be deleted at a later stage of development when TCR densities on these cells are increasing¹⁹. In conclusion, tolerance induction to the two antigens (LCMV, Mls^a) examined with the same transgenic receptor differed drastically. These findings indicate that T-cell tolerance by clonal deletion does not occur at one single discrete stage of T-cell development. □

Note added in proof: Since the submission of this manuscript, Berg *et al.*²⁰ have reported that $\alpha\beta$ TCR (V α_{11} , V β_3) transgenic Mls-2^a and -3^a mice exhibit deletion of CD4⁺CD8⁺ thymocytes, whereas β TCR V β_3 transgenic Mls-2^a and -3^a mice do not delete CD4⁺CD8⁺ thymocytes. Because the TCR density on thymocytes of $\alpha\beta$ -transgenic mice is higher than that in β -transgenic mice, it was argued that deletion of CD4⁺CD8⁺ thymocytes correlates with TCR density and maturation state. Because we found that the same CD4⁺CD8⁺ thymocytes are deleted in the LCMV-carrier but not in Mls^a mice, TCR density and maturation state alone cannot explain our findings, suggesting that affinity could also play a part. □

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***In vivo* priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine**

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CYTOTOXIC T lymphocytes (CTL) constitute an essential part of the immune response against viral infections¹. Such CTL recognize peptides derived from viral proteins together with major histocompatibility complex (MHC) class I molecules on the surface of infected cells^{2–4}, and usually require *in vivo* priming with infectious virus⁵. Here we report that synthetic viral peptides covalently linked to tripalmitoyl-S-glycerylcysteinyl-serine (P₃CSS) can efficiently prime influenza-virus-specific CTL *in vivo*. These lipopeptides are able to induce the same high-affinity CTL as does the infectious virus. Our data are not only relevant to vaccine development, but also have a bearing on basic immune processes leading to the transition of virgin T cells to activated effector cells *in vivo*, and to antigen presentation by MHC class I molecules.

The epitopes recognized by MHC class I-restricted, virus-specific CTL can be defined by short synthetic peptides (ref. 3; see also Fig. 1). These peptides are thought to bind to a groove on top of the MHC molecule, as indicated by its crystallographic structure⁴. CTL can recognize target cells incubated in picomolar concentrations of a given peptide (ref. 6; see also Fig. 2). Paradoxically, mice cannot be primed with the same peptide at any concentration tested (H. S. *et al.*, manuscript in preparation). An example for this failure is shown in Fig. 2a, d, g: BALB/c (H-2^d) mice were immunized with influenza nucleoprotein (NP) NP147–158 (R-) epitope (Fig. 1), which is the most efficient peptide to be recognized by H-2^d-restricted CTL specific for influenza nucleoprotein⁶. On stimulation of recipient spleen cells *in vitro*, no killing of peptide-incubated or virus-infected target cells was observed. By contrast, mice immunized with infectious influenza virus (Fig. 2b, e, h) responded well.

The failure of peptide to prime *in vivo* could be related to the non-physiological form of antigen presentation, because

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CTL usually recognize fragments derived from protein endogenously produced in the target cell^{7,8}, and/or due to a missing second signal, consisting of cytokines released by antigen-presenting cells⁹. We therefore attempted to prime virus-specific CTL *in vivo* with NP147-158 (R-) peptide conjugated to P₃CSS (see Fig. 1). The latter was synthesized according to the immunologically active N-terminal sequence of the principal lipoprotein of *Escherichia coli*, also known as Braun's lipoprotein¹⁰. P₃CSS mediates attachment to the cell membrane, internalization into the cytoplasm, and activates macrophages to secrete cytokines^{11,12}. Priming with the NP147-158(R-)/P₃CSS-conjugate was indeed efficient, producing a virus-specific CTL response as strong as that induced by priming with infectious virus (compare Fig. 2c, f, i with 2b, e, h). CTL priming was also efficient with two other P₃CSS-peptides (Fig. 3). One contained the influenza NP147-158 peptide, the other influenza NP365-380, which are recognized by H-2^d or H-2^b-restricted CTL, respectively^{6,13}. Figure 4a-c shows that only P₃CSS-[NP147-158 (R-)], but not Ser-Ser-[NP147-158 (R-)] or NP147-158(R-), is efficient for priming. Lipopeptide priming is MHC class I-restricted, as shown for two lipopeptides in Fig. 4d-i.

CTL primed *in vivo* with lipopeptide and restimulated *in vitro* with native peptide have a remarkable ability to recognize virus-infected cells. This is in marked contrast to those peptide-specific CTL obtained by the *in vitro* stimulation of spleen cells from unprimed mice at high cell density with viral peptide. Such

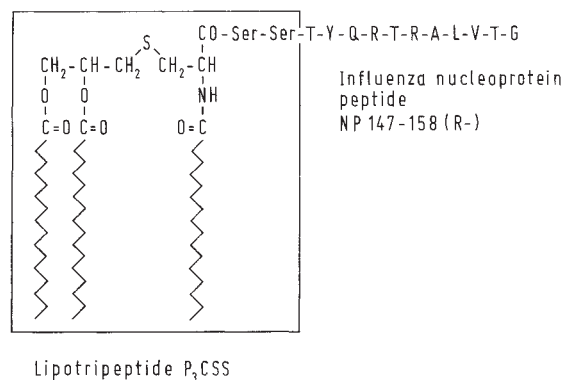


FIG. 1 Chemical formula of lipopeptide vaccine P₃CSS-[NP147-158 (R-)] of relative molecular mass (*M_r*) 2,332 (single-letter amino-acid code). Influenza NP peptide was synthesized by solid phase techniques on an Applied Biosystems 430A synthesizer using our own Fmoc program, Fmoc-L-amino acids and *p*-benzyloxybenzylalcohol-resin. The peptide was elongated with two additional serine residues followed by tripalmitoyl-S-glycerylcysteine (coupling procedures: (benzotriazolyl)tris-(dimethylamino)phosphonium hexafluorophosphate (BOP)/1-hydroxybenzotriazole (HOBt)), except for Gln coupled as symmetrical anhydride with diisopropylcarbodiimide (DIC), and P₃C coupled with DCC/HOBt. Peptide and lipopeptide were removed from the resin with trifluoroacetic acid containing 2.5% thioanisole and *p*-thiocresol (1 h at 25 °C, and for complete deprotection of Arg(2,2,5,7,8-pentamethylchroman-6-sulphonyl [Arg(Pmc)] additional treatment for 30 min at 50 °C). Amino-acid analysis, enantiomer analysis by gas-liquid chromatography on Chirasil-Val and (+)-FAB mass spectrometry proved the identity of peptide and the peptide portion of the lipopeptide. Purity of lipopeptide was more than 90% as analysed by HPLC. The amino-acid sequence outside the box represents the peptide reported to be most efficiently recognized by H-K^d-restricted influenza-specific CTL (ref. 6). This sequence is analogous to amino acids 147-158 of NP of influenza strain A PR/8/34 (PR8), except that the Arg at position 156 has been deleted. Additional lipopeptide vaccines were synthesized by coupling the lipotriptide P₃CSS as shown inside the box to influenza NP peptide of residues 147-158 (TYQRTRALVRTG), which is also recognized by H-K^d-restricted CTL, albeit with 1,000-fold less efficiency as compared with NP147-158 (R-) (ref. 6), or to influenza NP peptide of residues 365-380 (IASNENMET-MESSTLE) which is recognized by H-2^b-restricted CTL (ref. 13). *M_r* of P₃CSS-[NP147-158] is 2488, that of P₃CSS-[NP365-380], 2,853. Known biological activities of P₃CSS are described in refs. 10-12, 18, 20-22.

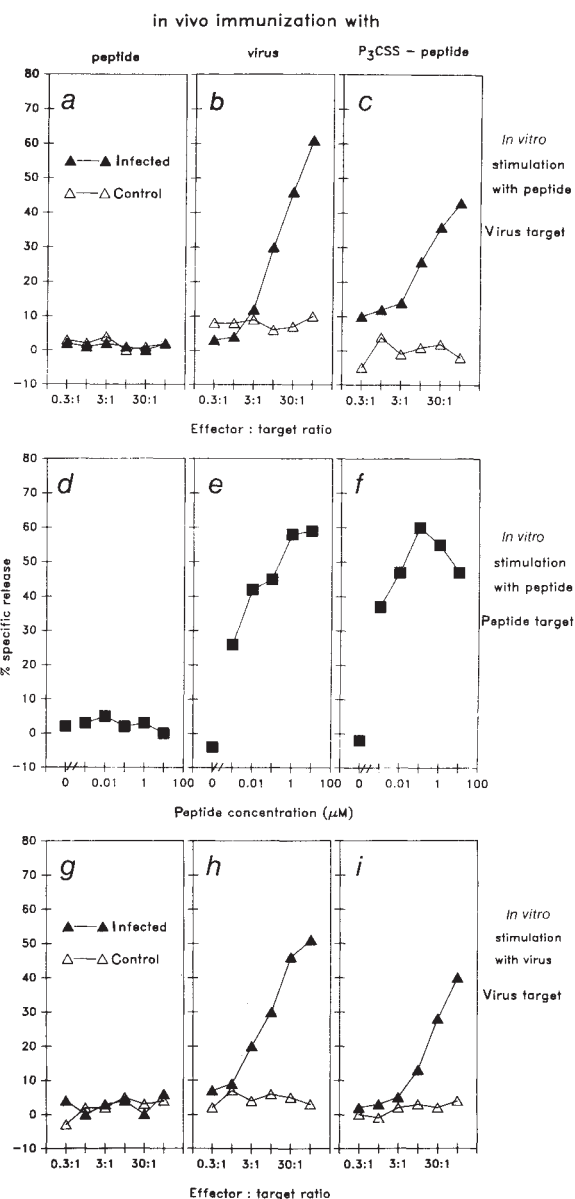


FIG. 2 CTL activity of spleen cells from BALB/c mice after immunization with peptide, virus, or P₃CSS-peptide. Adult BALB/c (H-2^d) mice were immunized with NP147-158 (R-) peptide (a, d, g), or with influenza virus (b, e, h) or with P₃CSS-peptide (c, f, i). After 6 days, recipient spleen cells were stimulated for 5 days against peptide (a-f) or strain PR8-infected syngeneic stimulator cells (g-i). a-c and g-i, CTL activity on untreated (△) or strain PR8-infected (▲) P815 (H-2^d) target cells. d-f, CTL activity on P815 cells, preincubated with titrated concentrations of peptide. Effector:target ratio in d-f, 30:1. a-g represent one experiment; h and i are from an independent experiment. Spontaneous ⁵¹Cr-release of target cells was between 10-20%. METHODS. Adult mice received intravenous injections of 8 × 10⁷ syngeneic spleen cells preincubated either with 1.6 μM NP147-158(R-) peptide (a, d, g) or with 160 μM of P₃CSS-[NP147-158(R-)] lipopeptide (c, f) or received 50 haemagglutinating units of influenza virus A strain PR/8/34 (PR8) (b, e, h) or 100 μg P₃CSS-[NP147-158 (R-)] lipopeptide (i). Volume of inocula was adjusted to 0.3 ml phosphate-buffered saline in all cases. Recipient spleen cells (2.5 × 10⁷) were stimulated *in vitro* in volumes of 10 ml α-minimal essential medium (MEM; Gibco) supplemented with 10% fetal calf serum, 2-mercaptoethanol, glutamine and antibiotics against 80 nM NP147-158(R-) peptide (a-f) or against 5 × 10⁶ PR8-infected, irradiated (2,000 rad) syngeneic spleen cells. CTL activity was determined in a standard ⁵¹Cr-release assay²³. Infection of stimulator and target cells was as previously described²⁴. Preincubation of target cells with peptide was for 30 min at 37 °C.

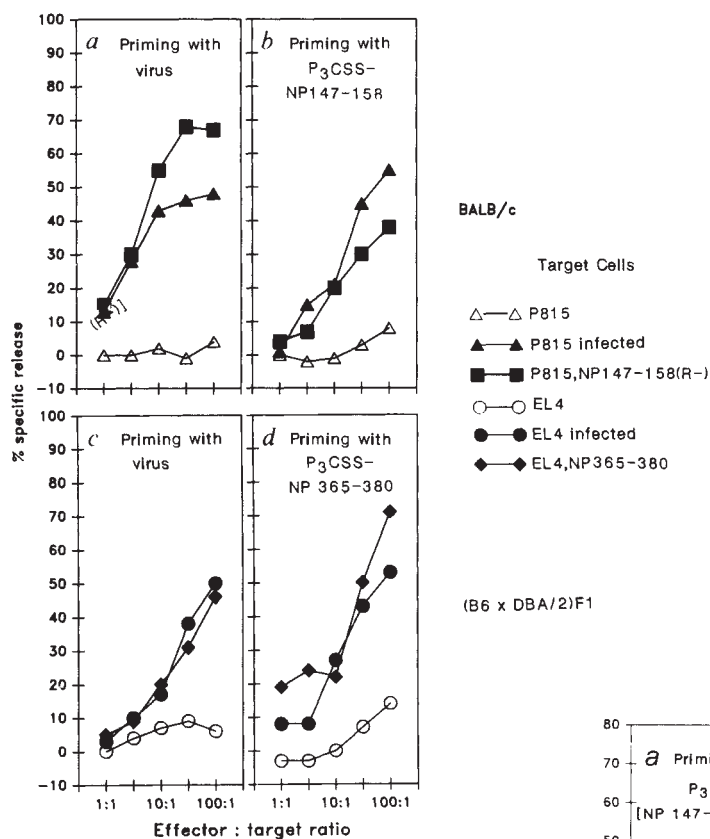
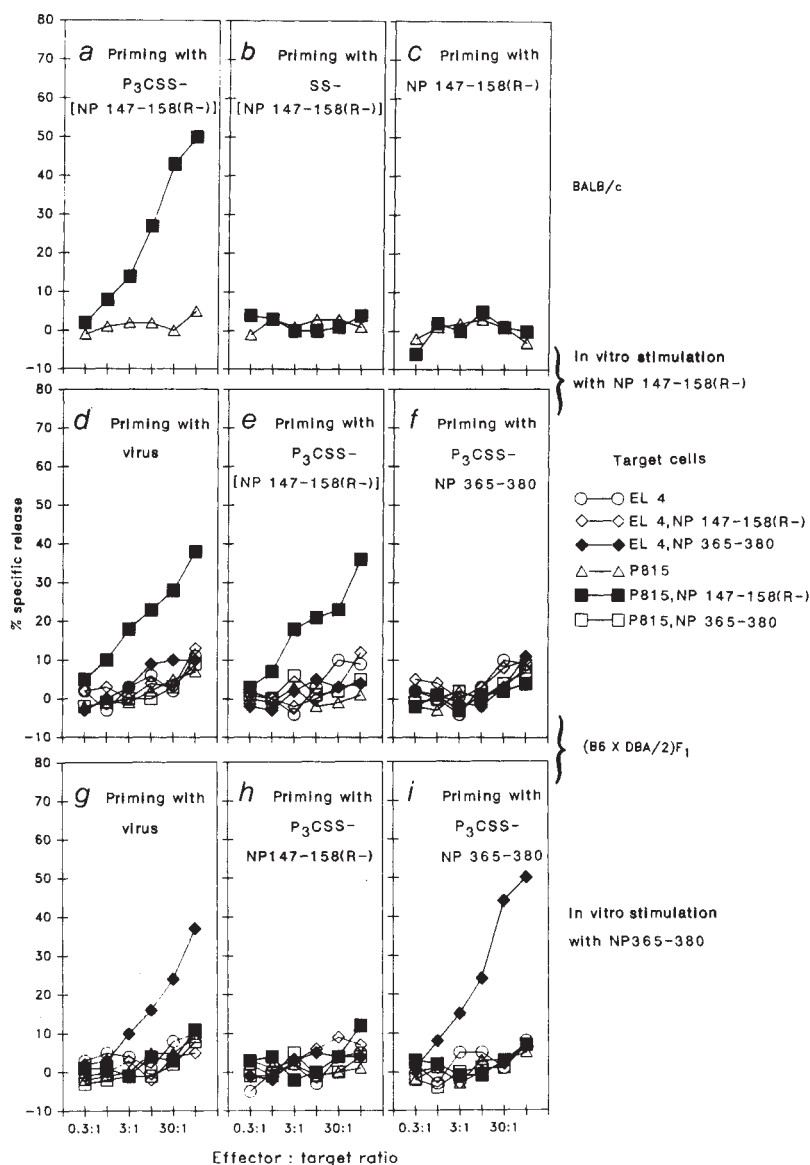


FIG. 3 CTL activity after priming of mice with P₃CSS-(NP147-158) or P₃CSS-(NP365-380) vaccines. BALB/c (a, b) or (B6 × DBA/2) F₁ (H-2^b × H-2^d) (c, d) mice were immunized with influenza virus (a, c) or with 100 µg P₃CSS-(NP147-158) (b) or with 100 µg of P₃CSS-(NP365-380) (d). After 6 days, recipient spleen cells were stimulated against 0.8 µM of NP147-158 peptide (a, b) or 0.8 µM NP365-380 peptide (c, d). CTL activity was determined on untreated P815 target cells, (△), PR8-infected P815 cells (▲), P815 cells preincubated with NP147-158 (R⁻) (■), untreated EL4 (H-2^p) targets (○), PR8-infected EL4 (●) or EL4 cells preincubated with NP365-380 peptide (◆). Spontaneous ⁵¹Cr release ranged between 11% and 25%.

METHODS. Preincubation of target cells with peptides was for 90 min at 37 °C using 0.8 µM of the respective peptide. Methods otherwise as in Fig. 2, legend.

FIG. 4 Specificity and MHC class I-restriction of lipopeptide-priming. BALB/c (a-c) or (B6 × DBA/2) F₁ (d-i) mice received injections of 100 µg P₃CSS-[NP147-158 (R⁻)] (a, e, h), 50 µg SS-[NP147-158 (R⁻)] (NP147-158 (R⁻) peptide elongated with two serine residues at the N terminus; M_r, 1,422) (b), 50 µg NP147-158 (R⁻) (M_r, 1,247) (c), PR8 virus (d, g), or 100 µg P₃CSS-NP365-380 (f, i). Six days after injections, recipient spleen cells were stimulated against NP147-158 (R⁻) peptide (a-f) or NP365-380 (g-i). CTL activity was determined on untreated P815 target cells (△), P815 cells preincubated with NP147-158(R⁻) (■), P815 cells preincubated with NP365-380 (□), untreated EL4 target cells (○), EL4 cells preincubated with NP147-158 (R⁻) (◇) or EL4 cells preincubated with NP365-380 (◆). Note that neither P815 nor EL4 express MHC class II molecules. Spontaneous ⁵¹Cr release of target cells was between 14% and 26%. Experiments performed as for Figs 2 and 3.



primary CTL do not, in general, recognize virus-infected cells, but do recognize peptide-incubated target cells. To explain this discrepancy it has been suggested that the affinity of these peptide-specific CTL primed *in vivo* is low compared with the affinity of CTL primed *in vivo* with infectious virus¹⁴. This assumes that virus-infected cells express the peptide-MHC complexes relevant for CTL recognition at very low density, as opposed to the density of such complexes presented by cells incubated with an exogenous source of peptide. Obviously, the lipopeptides that we used are able to induce the same high-affinity CTL as virus-infected cells do.

We do not know the exact mechanism of *in vivo* priming by P₃CSS-peptides. A trivial explanation would be based on the time of availability of the respective peptide preparation to be accessible for CTL; other possibilities could be based on the introduction and internalization of the conjugate into the normal MHC class I presentation pathway by means of membrane attachment^{14,15}, which has been suggested to be responsible for the occasional ability of complete proteins, or large fractions of them, to prime CTL *in vivo*^{16,17}. A third possible mechanism might depend on the activating effect that the P₃CSS anchor has on certain cells including macrophages, inducing the release of cytokines serving as differentiation factors for CTL⁹. The insertion of variations into the lipopeptide P₃C should establish which of these mechanisms are required for the transition of a virgin CTL *in vivo* to an active effector/memory cell, as well as for the introduction of a peptide into the MHC class I presentation pathway.

Our data show for the first time that virus-specific CTL, an important component of the immune response against viral infection, can be primed *in vivo* with a totally synthetic molecule, P₃CSS-peptide. Toxic effects of P₃CSS have not been observed *in vivo*¹⁸. A general problem limiting the use of peptide vaccines in outbred populations, however, is that T cells from MHC-different individuals recognize different peptides from viral proteins, always in conjunction with the respective self MHC (ref. 13). Thus, a universal synthetic vaccine to prime a population for CTL response against a given virus should contain all of the relevant epitopes recognized by T cells. Fulfilment of this requirement by using a mixture of lipopeptides seems not to be too far-fetched, given the relatively good accessibility of synthetic P₃CSS-peptide conjugates^{19,20}. P₃CSS conjugated to peptides seems to be especially suited for vaccine design, because the induction of neutralizing antibodies, the other principal contribution to viral immunity, can also be achieved with P₃CSS conjugated to the relevant epitopes^{18,20}. □

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T-cell tolerance by clonal anergy in transgenic mice with nonlymphoid expression of MHC class II I-E

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T-CELL reactivity to the class II major histocompatibility complex I-E antigen is associated with T-cell antigen receptors containing the V β gene segments V β 17a (ref. 1) and V β 5 (ref. 2). Mice expressing I-E with the normal tissue distribution (on B cells, macrophages, dendritic cells and thymic epithelium) induce tolerance to self I-E by clonal deletion in the thymus³. By contrast, we find that transgenic INS-I-E mice that express I-E on pancreatic β -cells, but not in the thymus or peripheral lymphoid organs, are tolerant to I-E (ref. 4) but have not deleted V β 5- and V β 17a-bearing T cells. Moreover, whereas T-cell populations from nontransgenic mice proliferate in response to receptor crosslinking with V β 5- and V β 17a-specific antibodies, T cells from INS-I-E mice do not. Thus, our experiments provide direct evidence that T-cell tolerance by clonal paralysis^{5–9} does occur during normal T-cell development *in vivo*.

To determine whether the tolerance to I-E in our INS-I-E mice resulted from deletion of I-E-reactive T cells, we used the V β 17a-specific monoclonal antibody KJ23 (ref. 1) and the MR9-4 monoclonal antibody that recognizes V β 5.1⁺ and V β 5.2⁺ T-cell receptors (TCR) (J. Bill, E. Palmer and O.K., manuscript submitted). INS-I-E transgenic mice analysed for the presence of V β 17a⁺ T cells were back-crossed several times to the SJL/J strain so that they were homozygous for the V β 17a gene. INS-I-E mice analysed for the presence of V β 5⁺ T cells were back-crossed several times to the C57BL/6 strain², such that they were positive for the V β 5 gene and negative for the V β 17a gene. Table 1 summarizes the results of immunofluorescent staining on peripheral lymph node cells of the INS-I-E mice. We found

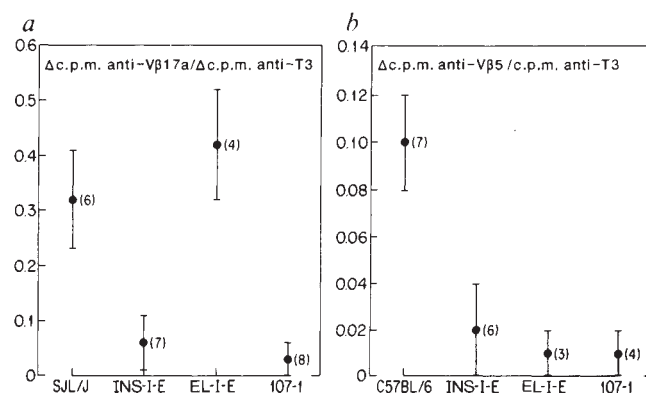


FIG. 1 Summary of T-cell proliferation induced by TCR crosslinking. Results are expressed as Δ c.p.m. anti-V β 17a/ Δ c.p.m. anti-T3 and Δ c.p.m. anti-V β 5/ Δ c.p.m. anti-T3. Values shown indicate $\bar{x} \pm$ s.e.m. obtained for individuals tested with both anti-V β and anti-T3 mAbs (n value given in parentheses).

TABLE 1 T Cells bearing I-E-specific receptors are not deleted in INS-I-E transgenic mice

I-E expression	Mouse	% $V\beta 17a^+$	Mouse	% $V\beta 5^+$
None	SVL/J	9.7 $\pm$ 1.0 (5)	C57BL/6	5.7 $\pm$ 0.9 (4)
Pancreatic β cells and kidney	INS-I-E	8.3 $\pm$ 0.5 (6)	INS-I-E	4.2 $\pm$ 0.6 (4)
Pancreatic acinar cells	EL-I-E	8.6 $\pm$ 0.9 (5)	EL-I-E	6.1 $\pm$ 0.1 (2)
Thymic epithelial cells	-1.4 kbE $_{\alpha}^d$ (v $^+$)	5.9 $\pm$ 0.3 (11) ¹¹	-1.4 kb E $_{\alpha}^d$ (v $^+$)	ND
Wild type	107-1	2.2 $\pm$ 0.5 (5)	107-1	0.1 $\pm$ 0.1 (2)

Percentage represents the average $\pm$ s.e.m. of n independent determinations (n value is given in parenthesis). Mice analysed for $V\beta 17a$ -bearing T cells were SVL/J strain, and transgenic INS-I-E, EL-I-E, -1.4 kbE $_{\alpha}^d$ (v $^+$) and 107-1 strains which were backcrossed to SVL/J to be homozygous for the $V\beta 17a$ allele. Only transgene-positive backcross progeny were used in our experiments. Lymph node T cells were prepared from these mice by treatment with J11d²² B-cell-specific monoclonal antibody followed by rabbit complement (Pel Freez, Brown Deer, Wisconsin). The frequency of positive cells is calculated as the percentage of cells staining with the KJ23 antibody (mouse IgG) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin as the secondary reagent, minus the percentage staining with second reagent alone. Mice analysed for $V\beta 5$ -bearing T cells were C57BL/6 or transgene $^+$ progeny of transgenic strains backcrossed to the B6 strain. Total lymph node cells were stained with MR9-4 antibody followed by FITC-goat anti-mouse IgG Fc-specific antibody (Jackson ImmunoResearch). The frequency of B cells detected with this secondary reagent is $<3\%$. Stained cells were analysed by flow cytometry on a Becton Dickinson FACStar. ND, Not determined.

no significant reduction in the frequency of $V\beta 17a^+$ T cells in these animals when compared with the SVL/J strain, and the intensity of staining between transgenic and SVL/J control T cells was comparable (data not shown), indicating that the density of TCR molecules per cell is similar. Furthermore, double-staining experiments reveal that the proportion of KJ23 $^+$ T cells that are CD4 $^+$ (65–70%) versus CD8 $^+$ (30–35%) were comparable in INS-I-E and SVL/J control mice. Transgenic 107-1 mice expressing I-E normally¹⁰ were analysed for $V\beta 17a$ after successive back-crossing to SVL/J. The low frequency of $V\beta 17a^+$ T cells observed (Table 1) reflects clonal elimination of these T cells, which is due to thymic expression of I-E¹¹. $V\beta 5^+$ lymph node cells were also not significantly reduced in INS-I-E mice compared with B6 controls (Table 1), whereas 107-1 mice on the C57BL/6 background have eliminated the $V\beta 5^+$ T cells. Thus, *in vivo* tolerance in INS-I-E mice is not due to clonal deletion of these I-E-reactive T-cell populations.

To determine whether T cells from the INS-I-E mice were clonally paralysed, we attempted to activate the I-E-specific T cells by receptor crosslinking. Normally, T cells are activated with antibodies recognizing their TCR in the presence of Fc receptor $^+$ accessory cells, or by insolubilized anti-TCR antibodies in the presence of accessory cells or soluble co-stimulatory signals^{12–14}. T cells that are anergic to I-E would not be expected to proliferate^{6,7}. Tables 2 and 3 show representative data generated with the $V\beta 17a$ - and $V\beta 5$ -specific antibodies. Stimulation of T cells with antibody 145-2C11 (ref. 15), which is specific for the T3 molecule of the T3/antigen-receptor complex, served as a positive control to demonstrate general responsiveness of the population.

T cells to which the KJ23 antibody was bound were cultured with T cell-depleted, spleen co-stimulator cells. Although proliferation could be induced in SVL/J control T cells (10 out of 11 individuals tested), INS-I-E transgenic T cells (7 out of 8 mice tested) showed little, if any, proliferation under the conditions described (Table 2). 107-1 mice, which have deleted most $V\beta 17a^+$ T cells, served as a negative control. Low levels of proliferation were sometimes observed from both INS-I-E and 107-1 T cells. This might be expected as not all $V\beta 17a^+$ T cells are I-E-reactive. A high percentage, but not all, of $V\beta 17a^+$ T-cell hybrids respond to I-E (ref. 1) and not all $V\beta 17a^+$ T cells are deleted in normal I-E $^+$ strains (ref. 11; and Table 1). Also, as individual $V\beta 17a^+$ T-cell hybrids differ in their reactivity to allogeneic forms of the I-E molecule¹¹, it is possible that not all $V\beta 17a^+$ T cells recognize the I-E expressed in INS-I-E mice (E $_{\alpha}^d$ E $_{\beta}$) equally well and so not all $V\beta 17a^+$ T cells will be tolerated.

Stimulation of spleen cells with the anti- $V\beta 5$ antibody MR9-4 insolubilized on anti-immunoglobulin coated wells (Table 3) revealed a positive response from C57BL/6 strain spleen cells (7 out of 8 individuals tested). In this case, insolubilized $V\beta 17a$ served as a negative control antibody. In contrast to B6 cells,

proliferation of INS-I-E T cells (5 out of 6 mice tested) was absent or greatly diminished. Only 1/6 individuals gave a sizeable response (Table 3, experiment 2).

Our data indicate that I-E-specific T cells are not clonally deleted in I-E tolerant INS-I-E mice. Instead, exposure to I-E on nonlymphoid cells *in vivo* apparently rendered the T cells anergic. Curiously, 30–35% of $V\beta 17a^+$ T cells and the vast majority of $V\beta 5^+$ T cells are CD4 $^+$ CD8 $^+$ in both normal and transgenic mice. Unlike normal CD8 $^+$ T cells (ref. 14; and O.K., unpublished results), these transgenic T cells are apparently unresponsive as well. Although CD8 $^+$ T cells are commonly restricted to class I, their use of particular $V\beta$ genes may confer sufficient reactivity to I-E to be paralysed.

We have also analysed EL-I-E transgenic mice that express I-E only on pancreatic acinar cells, are tolerant to I-E¹⁶ and

TABLE 2 INS-I-E transgenic T cells are not activated by anti- $V\beta 17a$ mAb

Expt	Responder	3 H]thymidine incorporation induced by TCR specific mAbs		Δ c.p.m.
		$V\beta 17a$ -specific mAb	T3-specific mAb	
		–	+	
		c.p.m. $\times 10^{-3}$		
1	SVL/J	3.3	24.9	21.6 (7.5)
	INS-I-E	0.9	1.1	0.2 (1.2)
	EL-I-E	2.7	17.5	14.8 (6.5)
	-1.4 kbE $_{\alpha}^d$ (v $^+$)	3.3	14.2	10.9 (4.3)
	107-1	0.4	0.3	-0.1 (0.8)
2	SVL/J	16.4	55.0	38.6 (3.3)
	INS-I-E	7.2	7.4	0.2 (1.0)
	107-1	8.1	16.4	8.3 (2.0)
	107-1	8.1	16.4	8.3 (2.0)
3	SVL/J	13.0	55.1	42.1 (4.2)
	INS-I-E	6.5	10.1	3.6 (1.6)
	EL-I-E	30.0	140.2	110.2 (4.7)
	-1.4 kbE $_{\alpha}^d$ (v $^+$)	8.1	46.9	38.8 (5.8)
4	SVL/J	11.5	67.9	56.4 (5.9)
	INS-I-E	14.7	21.0	6.3 (1.4)
	107-1	3.3	3.1	-0.2 (0.9)

Total c.p.m. $\times 10^{-3}$ or Δ c.p.m. are reported with stimulation index given in parenthesis. Lymph node T cells were prepared by treatment with J11d + rabbit complement in experiments 1 and 2, and L3T4 $^+$ T cells prepared by J11d and anti-Lyt 2 (53-6.7, rat IgG2a) + complement in experiments 3 and 4. These T cells were coated with KJ23 monoclonal antibody (mAb) by incubation with KJ23-containing ascites (diluted 1/10) for 30 min at 4°C. After washing to remove unbound antibody the cells were then plated at 2×10^6 responder cells per well in Costar 24-well plates (product plate number 3524). Syngeneic, mitomycin C-treated Fc receptor $^+$ splenic stimulator cells were depleted of T cells by anti-thy 1.2 (mouse IgM; New England Nuclear) plus complement and 2.5×10^6 cells were added per well. Cells were cultured in RPMI 1640 medium containing 2-mercaptoethanol, L-glutamine, penicillin and streptomycin, and 10% (v/v) FCS (Hyclone). On day 4, cells were transferred to Falcon 96-well (3072) plates (2×10^5 cells per well) and triplicate wells pulsed with 3 H]thymidine on days 4, 5, or 6 for the final 6 h of culture. Data reported are shown for day 5. Stimulation of all T cells with the anti-T3 mAb 145-2C11 reportedly occurs at a lower T-cell density with earlier kinetics in the presence of Fc receptor $^+$ accessory cells and was performed as a positive control¹⁵. Specifically, 1×10^5 responder cells and 2×10^5 T cell-depleted mitomycin C-treated stimulator cells were added to wells of a 96-well plate. Cells were cultured for 3 d in the presence of 145-2C11-containing cell culture supernatant with 3 H]thymidine added during the last 18 h of culture.

TABLE 3 INS-I-E transgenic T cells are not activated by anti-V β 5 monoclonal antibody

Expt	Responder	³ [H]thymidine incorporation induced by mAb specific for		
		V β 17a c.p.m. $\times 10^{-3}$	V β 5 c.p.m. $\times 10^{-3}$	T3 Δ c.p.m.
1	C57BL/6	0.2	19.5	19.3 (97.5)
	C57BL/6	0.3	39.2	38.9 (130.7)
	INS-I-E	3.5	3.9	0.4 (1.1)
	INS-I-E	3.5	3.2	-0.3 (0.9)
	107-1	1.0	4.3	3.3 (4.3)
2	C57BL/6	0.5	11.6	11.1 (23.2)
	INS-I-E	1.8	4.6	2.8 (2.6)
	INS-I-E	2.3	18.8	16.5 (8.2)
	EL-I-E	1.1	5.9	4.8 (4.9)
	107-1	1.0	1.2	0.2 (1.2)
3	C57BL/6	1.0	42.3	41.3 (42.3)
	EL-I-E	1.2	6.8	5.6 (5.7)
	107-1	1.7	0.7	-1.0 (0.4)

Data are expressed as total c.p.m. $\times 10^{-3}$ ³[H]thymidine incorporated or Δ c.p.m., with stimulation index given in parentheses. T-cell proliferation was induced by receptor crosslinking with mAb specific for V β 17a- or V β 5-encoded TCR. Falcon 96-well plates were coated with goat anti-mouse IgG (Jackson ImmunoResearch) at 20 μ g ml⁻² in PBS for 4 h at room temperature. After washing, TCR-specific mAbs were added (50 μ l undiluted cell culture supernatant or ascites diluted 1/100) for 2 h at room temperature, wells were washed again, and 2×10^5 spleen cells were added per well from transgenic mice backcrossed to C57BL/6. Cells were cultured for 5 d in RPMI 1640 medium supplemented as described in Table 2 legend, except that 1% (v/v) fresh normal mouse sera was added in experiment 1 and 10% (v/v) FCS was added in experiments 2 and 3. ³[H]thymidine was included for 6 h on the final day of culture. Stimulation with anti-T3 was carried out by culturing 1×10^5 spleen cells per well with 145-2C11 mAb cell-culture supernatant for 3 days; ³[H]thymidine was added during the final 18 h of culture.

have normal numbers of V β 17a and V β 5⁺ T cells (Table 1). In contrast to INS-I-E mice, EL-I-E T cells (4 out of 5 mice tested) proliferate in response to anti-V β 17a at levels comparable to SJL/J T cells. This difference between INS-I-E and EL-I-E V β 17a⁺ T cells (both lines back-crossed 4–6 times to SJL/J) indicates I-E expression in these two transgenic strains is not equivalent. As both lines express I-E at high levels^{4,16}, our data argue in favour of a qualitative difference in I-E^{17,18}. We suggest that V β 17a⁺ T cells do not recognize the particular I-E peptide complexes presented in EL-I-E mice. Although EL-I-E mice respond to anti-V β 17a at control levels, anti-V β 5 induced low levels of proliferation compared with B6 controls. Thus, V β 5⁺ and V β 17a⁺ T cells differ in their ability to be tolerized by the I-E presented in EL-I-E mice.

We have also examined T cells from the -1.4 kbE α (v⁺) line (which is a line carrying E α transgene with 1.4 kb of 5' flanking DNA and pBR322 vector sequence), which expresses I-E only on the thymus epithelium. These mice have only a partial deletion of V β 17a⁺ T cells (ref. 11, and reviewed in ref. 19) but they generate only a weak mixed lymphocyte response to I-E (ref. 10; and D.L., unpublished results). Interestingly, the V β 17a⁺ T cells that mature in these mice proliferate significantly when their I-E-specific TCR are crosslinked (Table 2).

A summary of the results obtained for T-cell proliferation induced by TCR crosslinking is shown in Fig. 1. Responses induced by anti-V β antibodies are expressed relative to those induced by anti-T3 antibodies to control for differences in the overall responsiveness of individual T-cell populations. As an additional control, we have stimulated T cells from C57BL/6 mice and transgenic lines backcrossed to the B6 strain with an anti-V β 8 (F23.1) antibody and find that they all respond comparably (data not shown).

In this report we show that INS-I-E and EL-I-E mice contain significant numbers of potentially I-E-reactive T cells, indicating that tolerance is not established by clonal deletion. The failure of these cells to respond to receptor crosslinking, using the experimental conditions described, indicates that clonal paralysis was induced instead. We are at present investigating whether or not there are conditions under which these cells will respond to TCR-mediated signalling. Our results parallel those described

for induction of T-cell unresponsiveness *in vitro*^{5–9}, presumably resulting from T-cell contact with major histocompatibility complex (MHC) borne by cells lacking full accessory cell function. Previous indications that clonal paralysis can occur *in vivo* include injection of chemically fixed accessory cells⁶ and MHC class II L-cell transfectants²⁰, and induction of *in vivo* tolerance to minor lymphocyte stimulatory antigens (MIs)²¹; our experiments, however, provide evidence that paralysis of T cells does occur during normal T-cell development in a transgenic mouse system. □

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Cell division in Malpighian tubule development in *D. melanogaster* is regulated by a single tip cell

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THE controlled production of cells by mitosis underlies the generation of pattern in a developing organism. Any particular structure is made up of a more or less constant number of cells^{1,2}, which results both from the size of the primordium and from the number of times these founder cells divide. Here I describe a mechanism that regulates cell division during the organogenesis of the insect kidney (Malpighian tubules) in *Drosophila melanogaster*. A single cell is shown to regulate the division of the other cells making up the tissue. This specialized cell, the tip cell, is set aside early in development and is distinctive both in its position and in its behaviour. Ablation of this cell arrests mitosis in the surrounding cells and prevents further growth. The presence of a tip cell in each tubule seems to be a feature of the development of Malpighian tubules in many insects, and its role in regulating cell division is similar to the control of germ-line mitoses by distal tip cells in the nematode gonad³.

In *Drosophila*, the primordium of the Malpighian tubules appears as four separate outpocketings of the prospective hindgut (Fig. 1a). These can first be seen in the extended germ band, 6.25 h after egg-laying. The primordium of each Malpighian tubule consists of 20–25 cells². The cells in these four primordia proliferate to establish the larval complement of 484 cells well before the end of embryogenesis². Further growth does not involve cell division but is achieved by enlargement of the cells, whose C number increases throughout late embryonic and larval

life^{1,4}. The proliferative activity of the primordial cells can be assessed by following their progress through the cell cycle. During DNA replication, cells incorporate added 5-bromodeoxyuridine (BUdR) and can then be labelled immunocytochemically to show that they have entered S phase⁵; the Malpighian tubules contain cells in S phase between 6.25 h and 8.75 h after egg-laying (Fig. 1*b*). The pattern of labelling with BUdR changes from a scattering of cells in S phase along the length of the tubules (Fig. 1*b*) to one in which only cells in the distal region of the tubules label (Fig. 1*c*). At 8.5 h after egg-laying, there are no nuclei in the S phase of the final mitosis in the proximal three-quarters of the tubule, whereas in the distal quarter, one to five nuclei (3.19 ± 1.47 , $n = 16$) have incor-

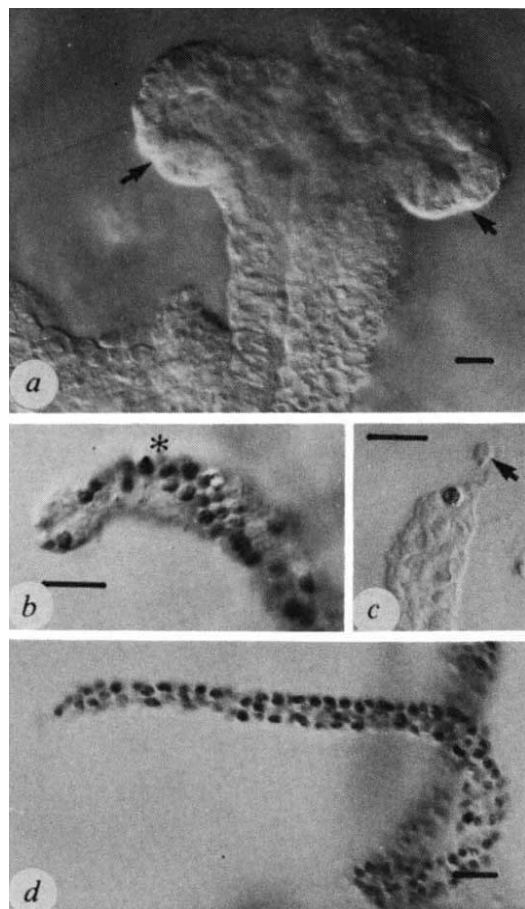


FIG. 1 The outgrowth of Malpighian tubules in the embryo of *D. melanogaster* and the patterns of cells in S phase revealed by the incorporation of BUdR. *a*, Proctodeum and primordia (arrowed) of two of the four Malpighian tubules dissected from an embryo 6.5 h after egg-laying. *b*, Tubule taken from an embryo incubated in BUdR starting 7.75 h after egg-laying. Labelled cells scattered along the length of the tubule can be seen but the unlabelled tip cell cannot. The asterisk indicates the region from which a cell was ablated as a control for tip-cell ablations (column E in Table 1). *c*, Malpighian tubule from an embryo incubated in BUdR starting 8.75 h after egg-laying. The only cells remaining in S phase are close to the tip cell (arrowed). *d*, Tubule taken from an embryo after incubation in BUdR which began 10 h after egg-laying. Almost all the cells have incorporated BUdR in the first endomitotic cycle. Scale bars, 10 μ m.

METHODS. *a*, The specimen was dissected unfixed and is viewed with Nomarski optics. *b-d*, The vitelline membrane was punctured and the embryos were incubated in a 40 μ M solution of BUdR (Sigma) in Schneider's medium (with L-glutamate) and minimal essential medium (GIBCO) for 15–30 min. They were then removed from the vitelline membrane in the same medium without BUdR, thoroughly rinsed and dissected before fixation for 30 min in Carnoy. Immunocytochemical localization of BUdR was as described in ref. 12.

porated BUdR. After S phase, labelled cells divide and the number of cells in each tubule rises from 20–25 at 6.25 h after egg-laying to 70 ± 4 at 7.75 h and to the mature number by 10.25 h. All the nuclei then enter S phase (Fig. 1*d*) in the first of several endomitotic cycles and the tubules increase in length by cell rearrangement (see Fig. 4).

A large cell at the distal tip of each Malpighian tubule becomes prominent (Fig. 2*a, b*) 1.5 h after the primordia are first seen, when cell division in the tubule is already underway. It is likely, however, that the tip cell is present and active before it becomes conspicuous. In *Rhodnius* the tip cell is apparent when the primordium first appears, coinciding with the onset of division. It does not incorporate BUdR (Fig. 2*c*) or divide (Fig. 2*d*), remains prominent throughout cell proliferation and tubule elongation, and is indistinguishable from its neighbours later in embryogenesis (after 14–16 h of development in *Drosophila*). Tip cells seem to be set apart from other tubule cells in that they do not cycle and divide during the stage of cell proliferation and their distal position is unchanged while the other cells are rearranging during tubule elongation. To investigate whether they have some special function in regulating tubule cell behaviour, the tip cells were ablated in one or two of the four tubules, the remaining intact tubules acting as internal controls. Removal of the tip cell has a dramatic effect on the further development of the tubules (Fig. 3). The operated tubules appear to have been arrested in development, remaining as clusters of cells protruding from the hindgut, whereas their intact siblings continue to grow and subsequently start to elongate, taking on the appearance of mature tubules. The number of cells per tubule are given in Table 1. If embryos are removed from the vitelline membrane and cultured, the Malpighian tubules grow and the number of cells in each increases. Ablation of the tip cell prevents division of the remaining Malpighian tubule cells (C), whereas culturing alone (B, D) or ablating cells in other parts of the tubules (E) has no effect on proliferation. Clearly the tip cells are required for the normal pattern of cell division in the development of the tubules, and their ablation results in premature arrest of mitosis in the remaining cells of the tubules. Further, the influence of the tip cell is confined to the tubule of which it is a part.

Regulation of cell division by tip cells is not confined to *Drosophila* embryos: tip cells are found in the developing Malpighian tubules of *Rhodnius prolixus* (Fig. 2*d*) and in *Schistocerca gregaria* and *Periplaneta americana* (unpublished observations). Furthermore, their influence resembles that of the distal tip cells (DTCs) in the gonad of the nematode, *Caenorhabditis elegans*³, in which ablation of the DTCs arrests normal mitotic division of germ cell precursors, which then enter meiosis without further cell proliferation. Moreover, control by the DTC can be limited to one ovotestis³ and the DTC signal is active

TABLE 1 Numbers of cells in each Malpighian tubule

Before culture	After culture			
	No ablation (control)	Tip-cell ablation		Control ablation
7.75-h Embryos		7.75-h Embryos + 6 h culture		
		Ablated tubules	Non-ablated tubules (internal control)	
70.4 ± 1.2 $n = 11$ (A)	105.6 ± 3 $n = 14$ (B)	75.3 ± 4 $n = 12$ (C)	107.6 ± 1.8 $n = 31$ (D)	103.3 ± 3.1 $n = 8$ (E)

Ablations and culturing were as described for Fig. 3. As a control (E), a cell other than the tip cell in the distal part of the tubule (Fig. 1*b*) was removed by suction with a glass micropipette. The numbers of cells were counted in dissected preparations after fixation and staining in toluidine blue. Values are means $\pm$ s.e., and n refers to the numbers of tubules counted. Analysis by a two-sample *T*-test shows that A and B, A and D, and C and D differ significantly ($P < 0.001$) and that A with C ($P = 0.29$), B with D ($P = 0.57$) and D with E ($P = 0.26$) are not significantly different.

over a short range: only the germ cell precursors closest to the DTC continue to divide mitotically, those further away enter meiosis and differentiate. In a similar way, as Malpighian tubules grow, cells further away from the tip cell cease to incorporate BVdR and divide. Although they both regulate mitosis in a neighbouring group of cells, in the nematode the DTCs persist through adulthood, maintaining a proliferating germ line, whereas the Malpighian tubule tip cells are active only over a

short period, resulting in a limited number of cells in each tubule.

It is possible that a common molecular mechanism underlies the similar functions of the two types of tip cell. In the nematode, mutations in the gene *glp-1*, which is expressed in germline cells, mimic the effect of DTC ablation⁶, suggesting that the *glp-1* product is involved in the interaction between the DTCs and the responding germline precursors. The amino-acid sequence deduced from the *glp-1* gene suggests that it encodes a transmem-

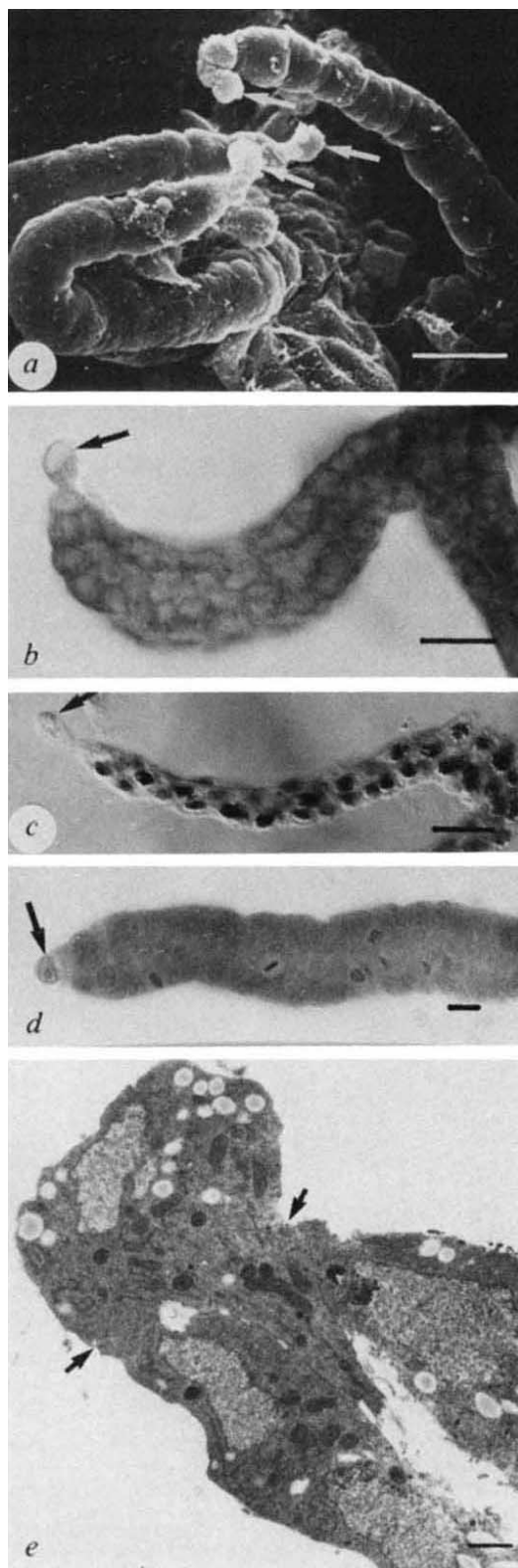


FIG. 2 Appearance and behaviour of the tip cell. *a*, Scanning electron micrograph of a 10-h embryo of *D. melanogaster* showing three of the Malpighian tubules with their prominent tip cells (arrows). *b*, Malpighian tubule from an embryo dissected 9.5 h after egg-laying. The tip cell (arrow) can be seen, protruding from the distal end of the tubule. *c*, Malpighian tubule taken from 9.5-h-old embryo, which had been exposed to BVdR for 15 min before fixation and immunocytochemical localization of the label as described above. Most of the cells in the tubules have incorporated BVdR, showing that they were in S phase during the period of exposure to BVdR, but the tip cell (arrowed) has not taken up any of the label. *d*, Distal region of a Malpighian tubule taken from a *Rhodnius* embryo. Although many cells in the tubule at this stage of development are in division, the tip cell (arrowed) is not. Scale bars, 10 µm. *e*, Electron micrograph of the distal region of a Malpighian tubule from a 9.5-h-old embryo. The tip cell does not form a cap over the end of the tubule, contact between cells is restricted to the lateral borders (arrows). Scale bar, 1 µm.

METHODS. *a*, Dissected embryos were fixed at 4 °C in Karnovsky's¹³ fixative, rinsed and dehydrated before critical-point drying and sputter-coating with gold. They were examined in a Philips SEM 505 microscope. *b*, Preparation stained with toluidine blue^{12,14}. *d*, Preparation fixed in Carnoy and stained in orcein. *e*, After dissection, tubules were fixed in 5% glutaraldehyde in phosphate buffer, pH 7.2, at 4 °C overnight. They were then washed and postfixed in 0.05% osmium tetroxide for 1 h and, after further washing, stained *en bloc* for 30 min in 2% uranyl acetate. Dehydration and embedding were by standard methods. Sections were cut on a Reichert OMU 2 ultramicrotome, stained with uranyl acetate and lead citrate, and viewed at 80 kV in a Philips EM 420.

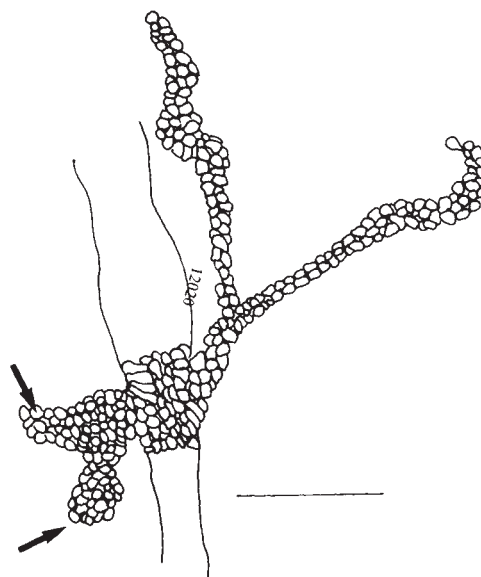


FIG. 3 Camera lucida drawing of Malpighian tubules from an embryo in which two of the four tip cells were ablated. The tubules without tip cells are arrowed. Both cell proliferation and tubule elongation are affected. Scale bar, 50 µm.

METHODS. Ablations were performed at the earliest time that the tip cell becomes prominent (7.75 h) and the embryo was cultured for a further 6 h in M3 medium¹⁵ at 25 °C before dissection and staining with toluidine blue. Ablation of the tip cell was carried out by removing it, by suction, with a glass micropipette.

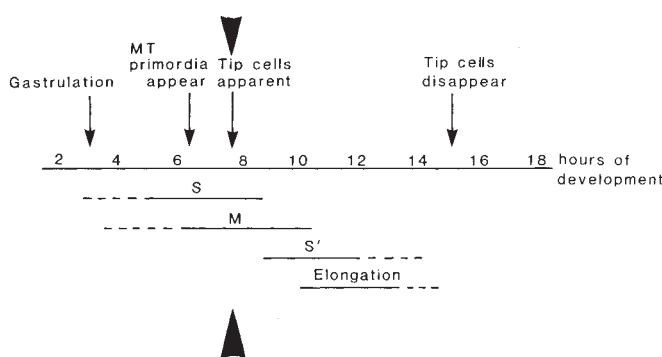


FIG. 4 Diagram to indicate the time when the tip cell was ablated (arrow-heads) in relation to some of the events in the development of the Malpighian tubules. Cells in the tubules are in S phase (S) before the divisions (M) that make up the mature number of cells in the tubules. A second period of DNA replication (S') is associated with the endomitotic cell cycles.

brane protein with an extracellular domain rich in epidermal growth factor-like repeats^{7,8}. Two models have been proposed^{9,10} for the interaction between DTCs and the *glp-1* protein (Glp-1). In the first, the DTCs release a signal that binds locally to a receptor, Glp-1, in the germ-cell membranes to activate mitosis. In the second, the *glp-1* protein directly stimulates mitosis but an interaction between Glp-1 and the extracellular matrix inactivates this function, so that cells in contact with the extracellular matrix enter meiosis. Thus the role of the tip cell is a passive one, in which, by shielding the outer membrane of the distal cells from the overlying extracellular matrix, it prevents the inactivation of mitosis. If there is a functional parallel between the two systems, the arrangement of cells in Malpighian tubules is not consistent with this second model because there is very little contact between the tip cells and the outer membranes of its neighbours (Fig. 2a, b, e). Electron microscopy also reveals that the extracellular matrix covers the external surface of all the cells in the tubule, including the tip cell and the cells adjacent to it (data not shown). The tip cell itself has the appearance of a cell actively involved in secretion: the endoplasmic reticulum is swollen, the cytoplasm is rich in Golgi and omega figures are found on the plasma membrane. There are similar specializations in the DTCs of the hermaphrodite gonad in the nematode, where they are essential for both mitosis and elongation of the ovotestis¹¹. These observations favour a model in which the tip cells have a more active role, perhaps in the secretion of extracellular matrix components, or of a signal, or both. Analysis of mutants affecting Malpighian tubule development in *Drosophila* should reveal further details of the mechanisms involved. □

Interaction of brain cytoplasmic dynein and MAP2 with a common sequence at the C terminus of tubulin

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TWO main types of microtubule-associated proteins (MAPs) have been identified in neuronal cells. The fibrous MAPs, including MAP2 and tau, serve to organize and regulate the assembly of microtubules. A second distinct class of force-producing MAPs, including kinesin¹, dynein²⁻⁴ and dynamin⁵, are involved in microtubule-based movement. These proteins are mechanochemical ATPases which seem to be responsible for the bidirectional transport of organelles and perhaps also the movement of chromosomes. Here we report that MAP2 inhibits microtubule gliding on dynein-coated coverslips, as well as the microtubule-activated ATPase of dynein, indicating that MAP2 and other fibrous MAPs could be important modulators of microtubule-based motility *in vivo*. By proteolytic modification of tubulin, we found that dynein interacts with microtubules at the C termini of α - and β -tubulin, the regions previously reported to be the sites for the interaction of MAP2^{6,7}. The use of site-directed antibodies implicates a small region of α - and β -tubulin, containing the sequence Glu-Gly-Glu-Glu, as the site of the interaction of dynein and MAP2 with the microtubule.

We observe only limited microtubule-gliding activity in partially purified dynein preparations, but this activity improves dramatically during further purification. Other than tubulin, the principal contaminants in the early stages of the dynein preparation are the fibrous MAPs. To test the effect of these MAPs on dynein force production, microtubules with or without MAPs were applied to dynein-coated coverslips in the presence of ATP (Fig. 1a). No motility was observed with the MAP-containing microtubules (lane 1), whereas >90% of the MAP-deficient microtubules (lane 2) showed gliding movement.

Inhibition of motility could result from steric hindrance of the interaction of the microtubule with dynein by the long (~100 nm) MAP arms⁸. Alternatively, inhibition could reflect competition between dynein and the fibrous MAPs for a common microtubule-binding site. To distinguish between these possibilities we examined the effect of purified MAP2 on brain dynein ATPase activity (Fig. 1b). MAP2 was found to inhibit the microtubule-activated component of the ATPase by 80%, with 50% inhibition occurring at a MAP2:tubulin-dimer molar ratio of 1:10. Inhibition as high as 97% was observed in other experiments. Chymotryptic microtubule-binding fragments of MAP2 also inhibited the dynein ATPase by up to 98% (data not shown). Together, these results indicate that dynein and MAP2 compete for common or overlapping binding sites on the microtubule surface.

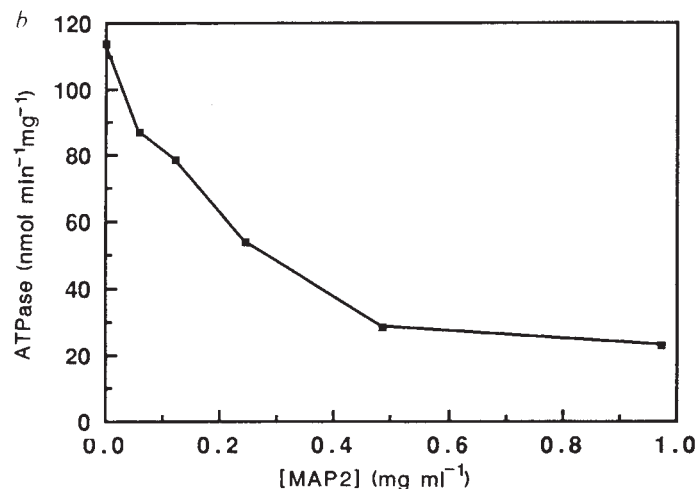
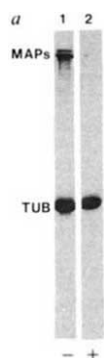
The analysis of MAP binding to tubulin fragments and synthetic peptides indicates that MAP2 and tau interact with the C termini of the tubulin subunits^{6,7}. Removal of the C termini of α - and β -tubulin with the protease subtilisin decreases the binding of MAP2 to microtubules⁶. To test whether dynein interacts with the same site on tubulin, we exposed taxol-stabilized microtubules to subtilisin and examined the effect of this treatment on the interaction with dynein. Proteolysis proceeded sequentially, first with the cleavage of β -tubulin to a species of relative molecular mass (M_r) apparently 4,000 (4K) less than that of β -tubulin (Fig. 2a, lane 2), and subsequently with a

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FIG. 1 Inhibition of dynein activity by MAPs. *a*, Polyacrylamide gel (8%) of MAP-containing and salt-extracted microtubules. The MAP-containing microtubules (lane 1) displayed no motility (–), whereas removal of the MAPs (lane 2) restored activity (+). *b*, Inhibition of the dynein ATPase by MAP2. Brain dynein ATPase assayed in the presence of 1 mg ml^{-1} microtubules was inhibited by increasing levels of purified MAP2. TUB, tubulin. **METHODS.** Brain cytoplasmic dynein (formerly referred to as MAP1C) was purified as previously described². Microtubule gliding was assayed as described in 50 mM KCl, 5 mM MgSO_4 , 0.5 mM EDTA, and 20 mM Tris KCl buffer, pH 7.6, containing 2 mM ATP (ref. 2). The microtubules were prepared by reversible assembly¹⁸, stabilized with $20 \mu\text{M}$ taxol (a gift of the NCI), extracted with 0.5 M NaCl for the MAP-depleted samples, and applied to the coverslip at a final concentration of 0.1 mg ml^{-1} . For the ATPase experiments, MAP2 was prepared from the heat-stable fraction of twice-cycled brain microtubules¹⁸, and separated from tau by chromatography on Biogel A5m (Biorad). The ATPase of brain



dynein (0.02 mg ml^{-1}) was assayed as previously described¹⁹ in the presence of 1 mg ml^{-1} taxol-stabilized microtubules prepared from DEAE-purified tubulin¹⁸. Increasing concentrations of MAP2 were added to the tubulin 15 min before the assay. Protein concentrations were determined with the bicinchoninic acid (BCA) reagent²⁰. The data shown represents the microtubule-activated ATPase after subtraction of the unstimulated ATPase.

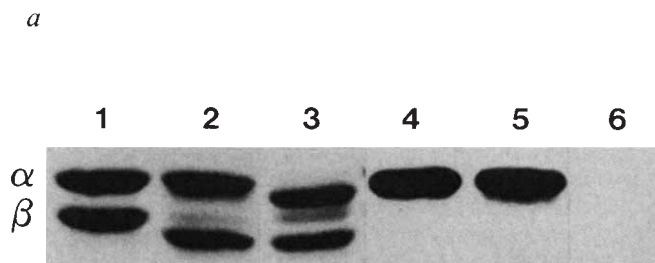


FIG. 2 The effect of subtilisin digestion of α - and β -tubulin on dynein ATPase activity. *a*, Electrophoretic analysis of digested tubulin. Taxol-stabilized microtubules were exposed to subtilisin for 0 (lanes 1, 4), 60 (lanes 2, 5) and 140 (lanes 3, 6) min and examined by Coomassie-blue staining (lanes 1–3) or immunoblotting with an antibody against the tyrosinated form of α -tubulin (anti-Tyr antibody, described in legend for Fig. 4). β -tubulin shifted to a position corresponding to a lower M_r by 60 min. (lane 2), whereas α -tubulin shifted to a lower M_r position by 140 min. The shift of α -tubulin coincided with the loss of anti-Tyr immunoreactivity (lane 6), indicating that the C terminus of α -tubulin was removed during subtilisin digestion. *b*, Effect of subtilisin digestion on microtubule activation of the dynein ATPase. The reduction in activity seems biphasic, with a relatively rapid decline corresponding to the digestion of β -tubulin, and a slower decline corresponding to the digestion of α -tubulin. *c*, Microtubule-concentration dependence of dynein ATPase using untreated taxol-stabilized microtubules (■), and microtubules which had been exposed to subtilisin for 140 min (○). The data in *b* and *c* are expressed after subtraction of the unstimulated ATPase. **METHODS.** Taxol-stabilized microtubules were used as the substrate for proteolytic modification, because proteolytic modification of tubulin subunits can result in aberrant polymers²¹. We found that the rates and patterns of fragmentation were the same as those reported when tubulin dimers were used as the substrate¹⁰, and thin-section electron microscopy confirmed that subtilisin had no detectable effect on microtubule morphology. DEAE-purified tubulin (5 mg ml^{-1}) in Tris buffer with KCl was assembled into microtubules with $50 \mu\text{M}$ taxol and digested with 1.2% (w/w) subtilisin (Boehringer) at 37°C . Digestion was terminated by the addition of PMSF to 5 mM. Electrophoresis was on 7% polyacrylamide gels containing 0.1% sodium lauryl sulphate (Sigma) at pH 9.2 for optimal resolution of α - and β -tubulin⁹. For ATPase assays, the tubulin samples were sedimented, washed with Tris KCl buffer containing 0.5 M NaCl, and used at a final concentration of 1.2 mg ml^{-1} .

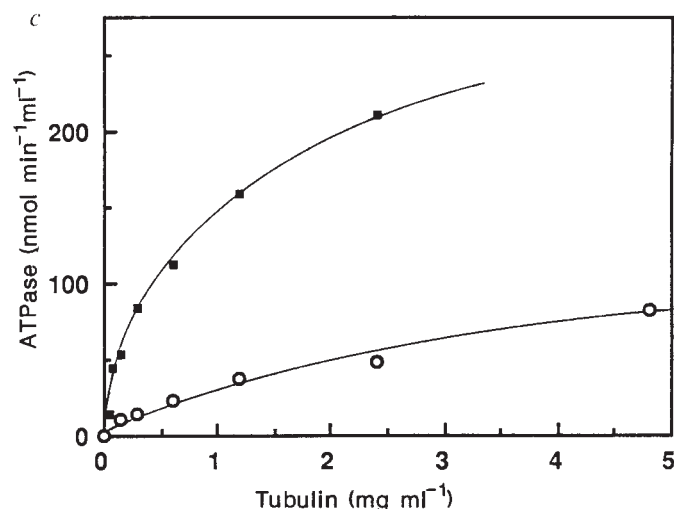
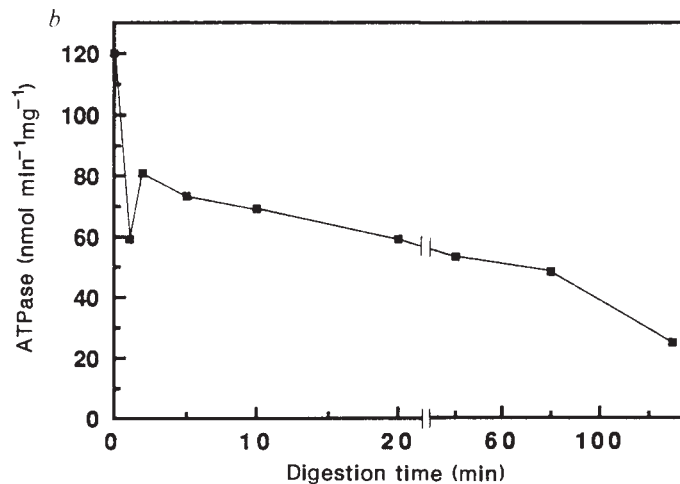


FIG. 3 The effect of thermolysin digestion of α -tubulin on dynein ATPase activity. *a*, Electrophoretic analysis of digested tubulin. Taxol-stabilized microtubules were exposed to 0 (lanes 1, 4), 0.1% (lanes 2, 5) and 1% thermolysin (lanes 3, 6) for 15 min at 37 °C and examined by Coomassie-blue staining (lanes 1–3) or immunoblotting with the anti-Tyr antibody (described in legend for Fig. 4). The shift of α -tubulin to a faster-migrating species did not result in a loss of reactivity (lanes 5, 6), indicating that the N terminus was removed during digestion. *b*, Microtubule-concentration dependence of dynein ATPase using untreated microtubules (■) and microtubules which has been exposed to 1% thermolysin for 15 min (○).

METHODS. Thermolysin (Boehringer) was used to digest taxol-microtubules in Tris buffer with KCl but no EDTA, and 5 mM EGTA was used to terminate the digestion. To identify the site of thermolysin action, α - and β -tubulin were separated by electrophoresis, transferred to Immobilon (Millipore), and the modified α -tubulin subjected to N-terminal microsequence analysis²². The resulting sequence, IGGGDDSF (single-letter amino-acid code) identified the site of cleavage as the amino side of Ile 42 (compare with ref. 14).

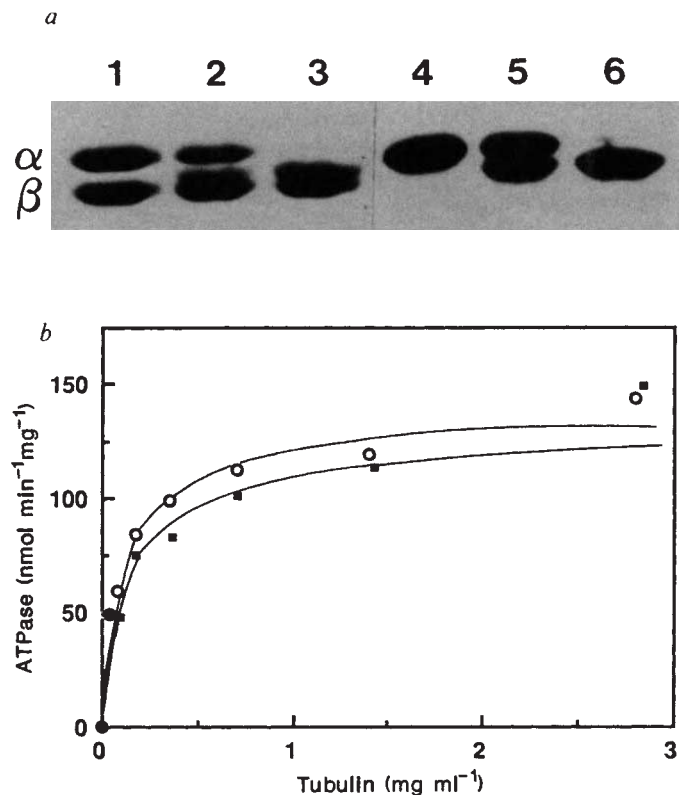
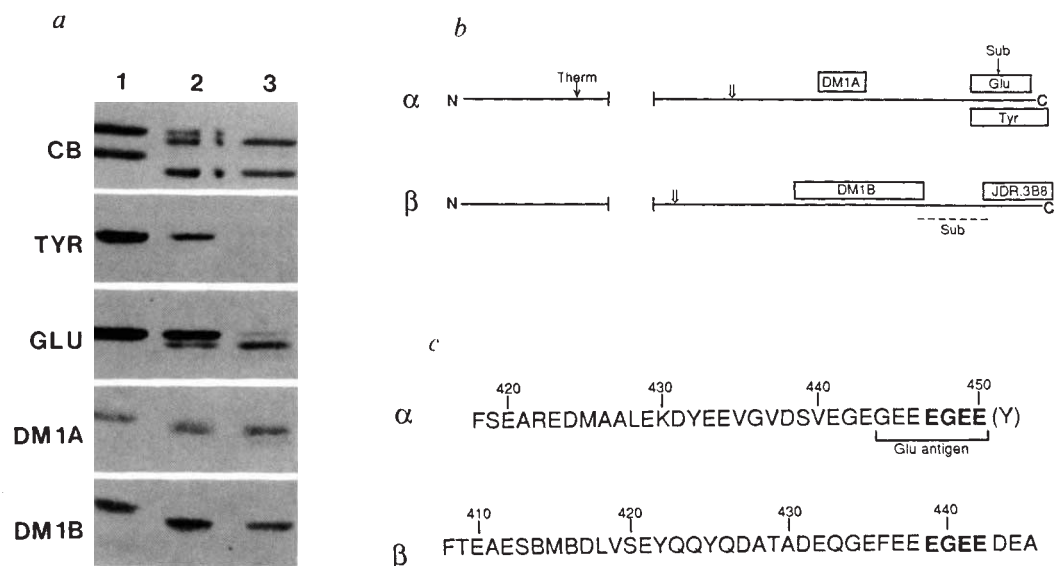


FIG. 4 Analysis of the site of subtilisin action. *a*, Immunoblot analysis of subtilisin-treated tubulin.

Microtubules composed of taxol-polymerized DEAE-purified tubulin (lane 1) were digested with 1.2% subtilisin for 60 (lane 2) and 140 (lane 3) min, and analysed by gel electrophoresis and immunoblotting. Abbreviations: CB, Coomassie blue; TYR, immunoblot using a site-directed antibody generated against the C-terminal sequence GEEEGEEY of tyrosinated α -tubulin¹¹; GLU, site-directed antibody generated against GEEEGEEY of detyrosinated α -tubulin¹¹; DM1A, monoclonal antibody (Amersham) reported to react most strongly with amino acids 426–430 of α -tubulin¹³; DM1B, monoclonal antibody (Amersham) whose epitope lies within amino acids 416–430 of β -tubulin¹³. The anti-Glu immunoreactivity was weaker with the subtilisin fragment than with intact α -tubulin, but was clearly positive.

The DM1A immunoreactivity (lane 2) resolved into two bands in other preparations. *b*, Schematic representation of cleavage sites and epitope locations within the C- and N-terminal regions of the tubulin subunits. The positions of the subtilisin (Sub) and thermolysin (Therm) cleavage sites deduced from the study described here are indicated, along with the previously proposed sites of subtilisin cleavage (open arrows). On the basis of the shift in electrophoretic mobility and amino-acid analysis of the subtilisin digestion products, the sites of subtilisin cleavage were previously assigned to Glu 417–Phe 418 and Glu 407–Phe 408 in α - and β -tubulin, respectively^{6,23}. Our results are consistent with the removal of a total of only nine acidic residues from the tubulin dimer as judged by native gel electrophoresis⁹. The antibody JDR.3B8 was generated against the sequence EGEEDEA, which corresponds to the C terminus of β -tubulin²⁴. We conclude that the subtilisin cleavage site in β -tubulin (dashed line) lies between the DM 1B and JDR.3B8 epitopes as the DM 1B epitope persists in subtilisin-



treated tubulin (*a*), whereas the JDR.3B8 epitope is destroyed (data not shown). We note that our results are consistent with the data of Littauer *et al.*, who found that MAP2 bound to peptides spanning the terminal 15 amino acids of β -tubulin⁷, although binding with peptides throughout α - and β -tubulin was also observed in their study. *c*, C-terminal sequence of porcine α - and β -tubulin^{14,25} beginning at the previously proposed subtilisin cleavage sites^{7,23} (single-letter amino-acid code). The sequence used to generate the anti-Glu antibody is bracketed. The sequence EGEE in bold comprises a significant portion of the anti-Glu epitope, and of the MAP2 and dynein interaction site as well.

METHODS. Subtilisin digestion and electrophoresis were as described in Fig. 2. The anti-Tyr and anti-Glu antibodies were used as unfractionated serum diluted 1:500, and the monoclonals were used as ascites fluid diluted 1:1,000. Blots were incubated with peroxidase-conjugated secondary antibodies (Cappel) and developed with 4-chloro-1-naphthol and hydrogen peroxide.

similar reduction in the apparent M_r of α -tubulin (lane 3)^{9,10}. The shift of α -tubulin to a faster-migrating species was coincident with the loss of immunoreactivity with an antibody to the C terminus of α -tubulin in its tyrosinated form¹¹ (anti-Tyr antibody, lane 6).

Figure 2b shows the effect of microtubules on the dynein ATPase during the time course of subtilisin digestion. The microtubule-activated ATPase activity was reduced by 80% after digestion of both α - and β -tubulin. Loss of ~50% of the activity occurred during the phase of β -tubulin digestion, whereas the remainder seemed to be due to the digestion of α -tubulin. Subtilisin digestion of both subunits also dramatically altered the concentration dependence of microtubule activation (Fig. 2c). Removal of the C terminus of β -tubulin alone increased the Michaelis constant, K_m , for microtubules from 0.53 mg ml⁻¹ (control) to 2.22 mg ml⁻¹ (data not shown). Thus, we conclude that the C termini of both α - and β -tubulin are directly involved in the activation of the dynein ATPase.

The residual microtubule activation after subtilisin treatment (Fig. 2c, lower curve) indicated that there could be an additional site of interaction with dynein. We treated taxol-microtubules with thermolysin, a protease that cleaves α -tubulin at a single site¹². There was a shift of α -tubulin to a position near that of intact β -tubulin (Fig. 3a, lane 3) corresponding to an apparent M_r loss of ~5K. The retention of C-terminal immunoreactivity (lane 6) indicated that, in contrast to subtilisin, the site of cleavage was near the N terminus. N-terminal microsequence analysis identified the site of thermolysin cleavage as the amino side of Ile 42 (see Fig. 3 legend). Cleavage of this portion of α -tubulin, which includes the site of tubulin acetylation, had no measurable effect on microtubule-activation of the dynein ATPase (Fig. 3b).

To define the site of dynein and MAP interaction more precisely, we probed the subtilisin digestion products using additional antibodies with epitopes near the C termini of α - and β -tubulin (Fig. 4a,b). Monoclonal antibodies DM1A and DM1B both reacted with the subtilisin-digested tubulin. The epitopes for these antibodies lie well within 4K of the C-termini of α - and β -tubulin¹³, respectively, indicating that the portion removed by subtilisin is overestimated by SDS-PAGE analysis.

Immunoreactivity with the anti-Glu antibody¹¹ also persisted after subtilisin digestion. This is particularly surprising because the antibody was prepared against the peptide GEEGEE (single-letter amino-acid code; G denotes Gly, E denotes Glu), which corresponds to the C terminus of α -tubulin in its dephosphorylated form¹¹. Because the anti-Glu antibody shows no reactivity with the tyrosinated form of α -tubulin, its epitope must include the C-terminal end of the peptide. Inspection of the α -tubulin sequence¹⁴ reveals that proteolytic removal of the four C-terminal amino acids of α -tubulin would expose the same tetrapeptide sequence, EGEE, found in the native dephosphorylated tubulin molecule (Fig. 4c). These results indicate that subtilisin removes a segment of α - and β -tubulin considerably smaller than that expected from SDS-PAGE analysis. Furthermore, considering the effect of subtilisin digestion on the interaction of MAPs and dynein with the microtubule, the sequence EGEE must be an important component of the MAP- and dynein-binding sites as well. Because there is some residual activation of dynein by subtilisin-treated microtubules (Fig. 2c), it is possible that the site of interaction could be larger, for example EEEGEE, or perhaps the tandem repeat EEEGEEGEE.

We note that despite the relative phylogenetic variability in the C terminus of tubulin, the sequences EGEE and EEEGEE are both well conserved (compare refs 15 and 16). The sequence EGEE appears in 15 of 21 metazoan α -tubulins, and EEEGEE or related sequences, such as DEGE (D denotes Asp), are found in the same tubulins. Twelve of twenty-four β -tubulins contain EEEGEE or EAAEE (A denotes Ala) close to their C termini, indicating that the site of MAP and dynein interaction in the β -subunit could be related to that in the α -subunit.

The existence of a common site for the interaction of the structural MAPs and dynein would seem to pose a problem for the cell. Are there distinct populations of MAP-containing and MAP-free microtubules in cells, the latter serving as the tracks for organelle transport? Trains of organelles have, in fact, been observed in lobster axons after release from a cold block, indicating that transport could occur along a subset of microtubules¹⁷. Alternatively, the MAPs could serve to regulate the rate or frequency of organelle movement, analogous to the regulation of myosin by tropomyosin. Further work is clearly needed to explore the implications of this novel role for MAPs, and to determine the effect of the C-terminal tyrosine on the interaction of microtubules with MAPs and the motor molecules. □

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Isolation of a gene regulated by hydrostatic pressure in a deep-sea bacterium

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BAROPHILIC bacteria inhabit the deep oceans, and the specific functional modifications and regulatory mechanisms which govern adaptation to hydrostatic pressure are beginning to be understood. For example, the rate of production of several proteins by some hydrothermal vent archaeobacteria¹ and the degree of saturation of membrane lipids in other deep-sea bacteria^{2–4} have been found to change as a result of cultivation at high pressure. We report here the cloning of gene, *ompH*, which encodes a major pressure-inducible protein of strain SS9, a gram-negative eubacterium isolated from a depth of 2.5 kilometres in the Sulu Sea. Messenger RNA encoded by *ompH* is expressed when cells are grown at 280 atm but not at 1 atm, indicating that transcription of the *ompH* gene is controlled by hydrostatic pressure. The function of the

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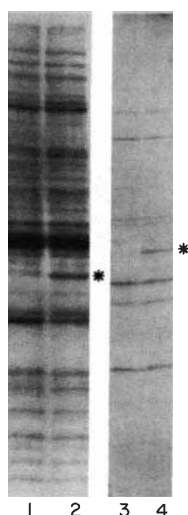


FIG. 1 Coomassie Blue R stained SDS-PAGE showing proteins produced by SS9 as a function of hydrostatic pressure. Lanes 1 and 2; equivalent amounts of total cellular proteins from cells grown at either 1 atm or 280 atm pressure. Lanes 3 and 4; Triton X-114 detergent preparation of hydrophobic membrane proteins from cells grown at 1 atm or 280 atm, respectively. Cells were grown in Difco 2216 Marine Broth at 15 °C to a cell density of $\sim 1 \times 10^7$ cells per ml using polyethylene culture bags, pressure vessels and accessory equipment which have been described previously⁸. SDS-PAGE was as previously described⁹. Triton X-114-associated proteins were prepared from 50-ml cultures using the procedure of Bourdier⁹, after first resuspending cells in 10 ml lysis buffer and incubating on ice for 30 min⁹. The 37K protein whose production is enhanced by pressure is shown by the asterisks.

OmpH protein in adaptation to high pressure and the use of the *ompH* gene in studying how bacteria sense and respond to pressure are discussed.

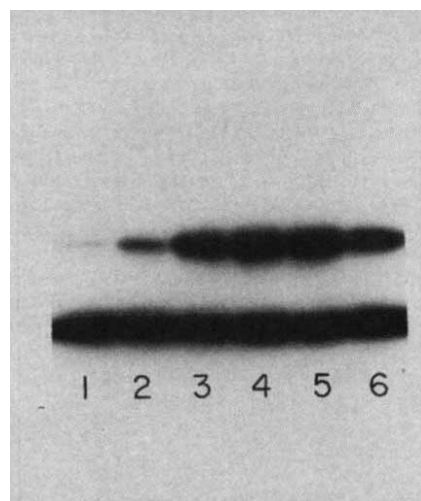
The optimum pressure for cultivation of strain SS9 is 280 atm (E. F. Delong and A.A.Y., manuscript in preparation), but the bacterium is not an obligate barophile and can also be grown at pressures as low as 1 atm, and at temperatures as high as 23 °C, which makes it amenable to manipulations with the techniques of molecular biology and microbial genetics. The responses of SS9 to growth at high pressure were surveyed by comparing proteins synthesized at high and low pressures. SDS-PAGE of total protein from SS9 is shown in Fig. 1; lane 1 contains protein from cells grown at 1 atm and lane 2 from cells

FIG. 2 Western PAGE analysis showing regulation of OmpH production by pressure. Equivalent amounts of SS9 whole cell proteins were separated by SDS-PAGE, electroblotted onto Nytran filters and subsequently reacted with antisera 109 and 111. 109 is used as a control because it recognizes a protein whose production is not regulated by pressure. From left to right are cells grown up at increasing pressures from 1 atm to 280 atm in 70-atm increments. OmpH is shown by the arrow. Cells were grown as described in Fig. 1. Western blotting was as described in ref. 9.

grown at 280 atm. The pattern of protein synthesis at the different pressures is similar except for the presence at 280 atm of a prominent protein of relative molecular mass (M_r) 37,000 (37K). The 37K protein is relatively abundant, and its presence in cell fractions obtained by detergent extractions indicate localization to the outer membrane. For example, the 37K protein is found in a hydrophobic membrane protein fraction prepared by Triton X-114 extraction of SS9 (Fig. 1; lanes 3 and 4) and in an outer membrane protein fraction prepared by Sarkosyl extraction (data not shown). The 37K protein is designated OmpH (outer-membrane protein, high pressure). The denatured OmpH protein was purified from Triton X-114 extracts by excision of the band from preparative SDS-PAGE. This preparation was used to prepare high-titre polyclonal antisera against OmpH. The resulting rabbit immune serum (number 111) specifically recognized the OmpH protein in western blot PAGE analyses. Antisera number 111 was used to measure OmpH production as a function of pressure. Whole-cell extracts from cells grown under different pressures showed up to a 70-fold induction at 280 atm of the OmpH protein. These results are based upon autoradiograph laser densitometry as well as gamma counting of ¹²⁵I-iodine-labelled protein-A western blots. A profile of the production of OmpH with cells grown at increasing pressure increments of 70 atm at 15 °C is shown in Fig. 2.

The *ompH* gene was cloned from an expression gene library of SS9 DNA in *Escherichia coli*. Specifically, partially *Sau*3A-restricted 2-4-kb SS9 chromosomal DNA was cloned into the expression plasmid pDOC55 (ref. 5), and expression from the vector's *lac* promoter was then induced with isopropyl β -D-thiogalactopyranoside (IPTG). One transformant out of 10,000 was immunopositive. Plasmid DNA isolated from a representative clone conferred immunoreactivity to 100% of recipient *E. coli* upon retransformation. Western PAGE confirmed that this clone, designated pDB500, produced an immunoreactive protein of the same size as OmpH. A second independent approach confirmed that pDB500 encodes the *ompH* gene. 100 picomoles of purified OmpH was electroblotted onto a polyvinylidene difluoride membrane, and the sequence of the first 20 N-terminal amino acids was determined. From this amino-acid sequence a corresponding mixed oligonucleotide probe (ompH-1) was designed. ³²P-end-labelled ompH-1 selectively hybridized to a particular region of the pDB500 plasmid containing SS9 DNA. In addition, the 5' end of the *ompH* gene was localized by mapping the site of ompH-1 binding (Fig. 3).

The *ompH* gene was further localized on the DNA clone by subcloning into the broad host range incP1 plasmid pRK415 (ref. 6). A *Kpn*I-*Hind*III pDB500 fragment which contains the SS9 insert from pDB500 and flanking polylinker sequences was cloned into *Kpn*I- and *Hind*III-restricted pRK415 to generate



pDB501. Transposon mutagenesis using Tn5 was used to localize the *ompH* gene on pDB501. Transposon insertions which disrupted OmpH production, measured by western blotting, mapped from 0.05 to 1.05 kilobases (kb) from the unique *Sst*I site as shown in Fig. 3. Those Tn5 insertions flanking *ompH* which did not affect OmpH production were located 1.65 kb apart. Therefore, the size of the *ompH* gene based upon Tn5 mutagenesis is between 1.0 and 1.65 kb in length. These results are consistent with the size of the OmpH protein, which is 37K. Tn5 insertions numbers 11 and 27 resulted in an immunoreactive OmpH protein truncated by ~3K and are therefore located approximately 100 base pairs (bp) from the 3' end of *ompH*.

Is transcription of the *ompH* gene regulated by pressure? RNA was isolated from SS9 cells grown at low and high pressure. Equivalent amounts of RNA were then electrophoresed in formaldehyde-formamide agarose, blotted and subjected to Northern blot analyses using pDB500 as the hybridization probe. As can be seen in Fig. 4, a single transcript of ~1.4 kb in length is

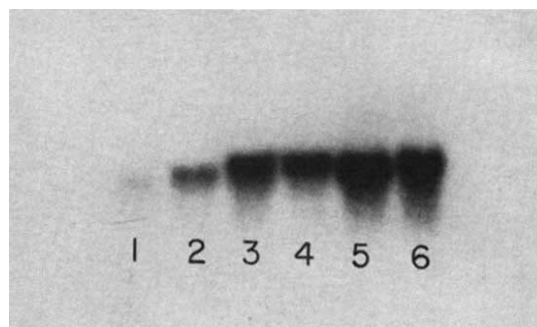


FIG. 4 Northern blot analysis of *ompH* message abundance as a function of pressure. Cells were grown as described in Fig. 1 and their total RNA purified, electrophoresed and blotted¹⁴. RNA (10 µg) was loaded into each lane. From left to right is RNA from cells grown at 1–280 atm in 70-atm increments. pDB500 served as the hybridization probe.

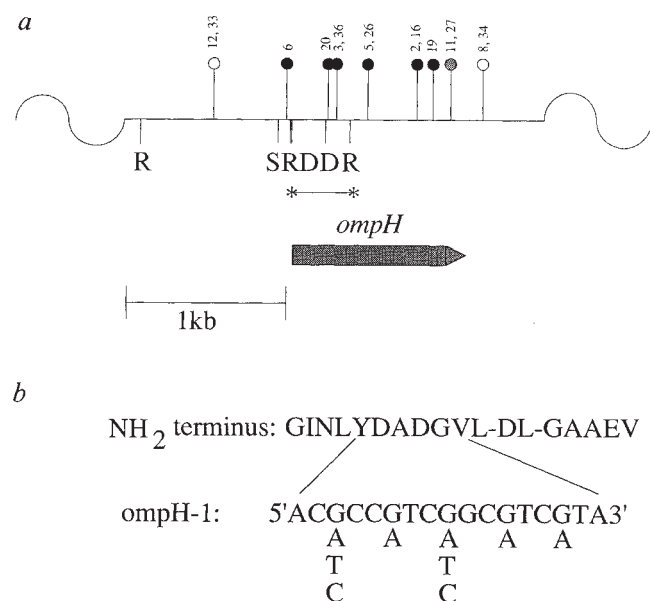


FIG. 3 Cloning and localization of *ompH*. *a*, Restriction-endonuclease map of *ompH* containing insert in pRK415 to generate pDB501. R = *Eco*RI, S = *Sst*I, D = *Dra*I. The positions of various numbered Tn5 insertions are shown by symbols above the restriction map. Closed circles represent serum 111 immunonegative derivatives of pDB501; open circles represent Tn5 insertions in pDB501 which retain the OmpH-immunopositive phenotype. The gray symbol represents the position of two Tn5 insertions which yielded a truncated immunopositive OmpH product. Tn5 mutagenesis was performed as previously described¹¹. A 1-kb size marker is present in the lower left corner. *b*, Amino-terminal sequence of OmpH and ompH-1 oligonucleotide design. SDS-PAGE-separated Triton X-114 membrane protein preparations containing OmpH were electroblotted and OmpH stained with Coomassie Blue R, cut out from the polyvinylidene difluoride membrane and sequenced using an Applied Biosystems 477A gas-phase protein sequencer as described by LeGendre and Matsudaira¹². The first 20 amino-terminal amino acids are shown as well as the sequence of ompH-1, a mixed oligonucleotide probe derived from amino acids 5–10 reading in from the amino terminus of OmpH. Dashes amongst the one letter amino-acid designations represent the positions of amino acids for which an unambiguous amino-acid assignment could not be made. Oligonucleotide synthesis was performed using an Applied Biosystems 380A DNA synthesizer. The oligonucleotide was end-labelled using T4 polynucleotide kinase and used in DNA hybridization experiments with plasmid DNA bound to Nytran membranes as described¹³. The ompH-1 *Eco*RI hybridizing fragment from pDB501 is shown as a bar with asterisks. The location and 5' to 3' orientation of the *ompH* gene based upon Tn5 mutagenesis and ompH-1 binding is also shown.

detected. As with OmpH protein production, the abundance of the *ompH* transcript increased with pressure up to about 280 atm. Based on laser densitometry and ³²P quantitation, this increase in *ompH* transcript abundance was at least tenfold. These results indicate that pressure influences *ompH* expression, probably at the transcription level of *ompH*, although we cannot exclude the possibility that pressure could be influencing the stability of the *ompH* messenger RNA.

The DNA sequence of the cloned *ompH* gene is being determined, and the derived amino-acid sequence of a part of the OmpH protein is available for comparison to the sequences of other proteins. Preliminary amino-acid sequence comparison indicates that OmpH is related to the *Neisseria gonorrhoeae* major outer-membrane porin protein⁷. This class of proteins form channels for the diffusion of molecules like amino acids and sugars into the periplasmic space. We speculate that porin channels which function at low pressure could be perturbed by high pressure which would necessitate production of a new porin molecule such as OmpH. The ability to adapt to changes in pressure suggests the existence of a genetic programme which couples sensing of pressure to production of appropriate activities. Our discovery that expression of *ompH* is responsive to pressure is direct evidence for such a programme. We can now proceed to identify components of the pressure-sensing circuitry, possibly including a pressure-sensitive repressor or a pressure-dependent activator of gene transcription which controls *ompH* expression. □

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ED: pulsed electrophoresis instrument

D. C. Schwartz, L. C. Smith, M. Baker and M. Hsu

Addressing the problems associated with commercial instrumentation for pulsed-field gel electrophoresis, the authors describe a versatile instrument that you can assemble yourself with considerable cost savings.

PULSED-FIELD gel electrophoresis (PFGE) size resolves DNA molecules of almost a millimetre in length through the use of pulsed electric fields, which selectively modulate mobilities in a size-dependent fashion^{1,2}. This tremendous resolving power of PFGE enabled the extension of the general experimental repertoire of molecular biology to chromosomally sized DNA molecules. Fortunately, the exact choreography that large molecules follow during conformational contortions induced by squeezing through relatively small gel pores is rapidly being elucidated by experimental^{3,4} as well as theoretical⁵⁻⁷ approaches.

The pulsed electrophoresis effect has been utilized by a variety of instruments (CHEF⁸, TAFE⁹, PACE¹⁰, OPHAGE¹¹ and rotating electrode/gel¹²) to increase the size resolution of both large and small DNA molecules. However, much of the published and commercial instrumentation compromises resolution, runs small gels, is unnecessarily complicated (leading to unreliability), and is costly. To meet the need for a simple, inexpensive, versatile pulsed electrophoresis instrument, we designed ED (electrophoresis device). ED runs large gels with straight lanes, permits field angle variation (the angle between applied electrical fields) and can accommodate field gradients if desired.

Instrument set-up

The schematic diagram of ED (Fig. 1) depicts a simple plexiglas tank with a removable bottom plate, which facilitates new electrode array configurations. An electrode array consists of a series of plexiglas pegs, each strung with a piece of platinum wire, connected to a diode and finally to a bus wire. This discrete electrode-diode system^{2,4} minimizes field interaction of the unpowered spectator electrodes and eliminates the need for cumbersome switching gear. The discrete electrode arrays produce a uniform field, nearby, thus emulating a continuous electrode such as a long platinum wire. Diodes are directly wired to each electrode to prevent significant conduction of the unpowered electrodes, which would contribute heavily to field distortion when the complementary electrode set is powered.

The electrode arrays are movable in the x-y axis of the tank and, thus, new field angles can be created by simply removing the bottom plate and drilling appropriate holes for the electrode pegs. A set of convenient holes are normally present to

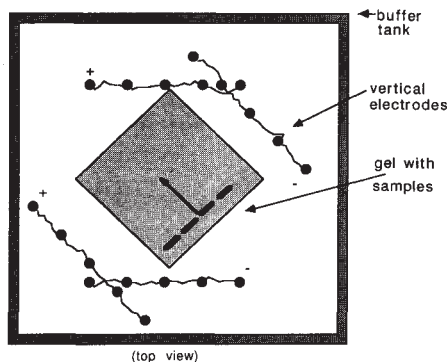


FIG. 1 Schematic diagram of ED electrophoresis tank showing plexiglas tank with four discrete diode electrode arrays. Dots: individual vertical electrodes each containing a diode; curly line: bus wire; + and -: polarity of each electrode array; arrow: net band motion; broken line: sample wells.

accommodate conventional arrangements. Field gradients can also be created by placing one or two electrodes opposite an entire array. Although field gradients can sharpen bands a little, the accompanying distortion can, however, make it difficult to interpret results.

Buffer circulation is facilitated by manifolds located at opposite ends of the instrument (not shown). A simple heat exchanger can be constructed from a modified spiral condenser, connected to a refrigerated bath for careful temperature control or, more economically, to running tap water.

As with many pulsed electrophoresis instruments, the field direction is periodically changed between the two sets of diode arrays, and the use of a voltage-regulated power supply assures that the potentials created across the gel are clamped to a set value. Switching equipment can be simply and inexpensively built in-house, consisting of a Chronrol programmable timer (Lindberg Enterprises, San Diego, California) or an Adtron data generating board (Gilbert, Arizona) interfaced to a heavy-duty a.c. relay. Alternatively, commercial units are available. Power supplies, purchased from companies such as Electronic Measurements (Neptune, New Jersey) are inexpensive, reliable and can power 5-10 units simultaneously due to their high amperage rating.

Separations achieved

There are several advantages to using ED, which usually accommodates gels that are 20 × 20 cm: many samples can be loaded at the same time, and the large running

area provides room for a wide range of sizes to be run out simultaneously. This is shown in Fig. 2, where an *Xho*I restriction enzyme digest of gel inserts^{2,5} of *Saccharomyces cerevisiae* DNA displays a separation range from 3 kb to over 1,000 kb, with sufficient space for excising bands without contamination from neighbouring bands.

A key feature of the pulsed electrophoresis effect is that pulse-time variation selectively resolves different size ranges^{1,2,5}, and the optimum pulse time generally varies with size so that larger molecules are better resolved using longer pulse times. This effect is demonstrated in Fig. 3. These gels were run out for a long time

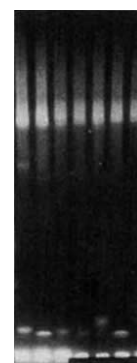


FIG. 2 Restriction enzyme digest of *Saccharomyces cerevisiae*. *Xho*I digests⁵ of DNA from various strains of *S. cerevisiae* resolved using ED (120° field angle) with the origin at the bottom of the photograph. Gel length 20 cm, with top bands sized at 3 kb and the bottom bands ~ 1 megabase. Running conditions: 1.0% agarose, tris-borate-EDTA buffer, 5 V cm⁻¹ field strength, pulse times of 100 seconds, 48 hours and 35 seconds, 24 hours.

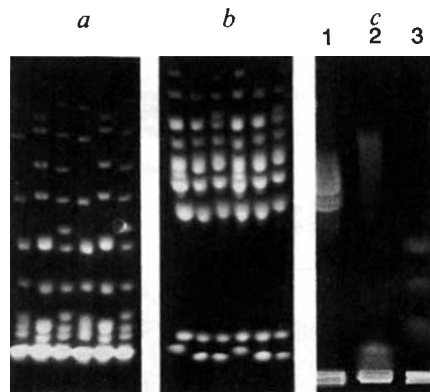


FIG. 3 Separations vary with pulse time. a, Same yeast DNA samples and running conditions as Fig. 2, band sizes range from 200 kb (top) to 2,500 kb (bottom); b, Same conditions and sample loading as a, except pulse times are 200 seconds, 48 hours, 30 seconds, 24 hours; c, Large DNA molecule resolution. Samples loaded are *Saccharomyces cerevisiae* (lane 1), *Drosophila melanogaster* (Schneider's line, lane 2), and *Schizosaccharomyces pombe* (lane 3). *S. pombe* chromosome sizes are 3, 5 and 7 megabases. Same conditions as a, except field strength is 3 V cm⁻¹ and running time is 96 hours.

to emphasize strain-dependent chromosomal size polymorphisms for many of the chromosomes. The very large chromosomal DNA molecules of *Schizosaccharomyces pombe* require long pulse times and are shown separated in Fig. 3c. As with most pulsed electrophoresis instruments, the running time is generally not a function of instrumentation, so that time can be minimized by increasing the field strength and decreasing the gel concentration, particularly when using high-strength agarose.

Other approaches use a series of strategically designed pulse trains to simultaneously open up selected windows of size resolution or field angles approaching 90°. Whatever the approach, however, compromises are always made depending upon the ultimate resolution desired, or the time allotted, particularly if separate bands must be excised for other experiments or easily visualized on a hybridization membrane.

To ensure that ED remains an inexpensive instrument, shop-ready plans are available upon request, which enable researchers either to construct the instrument themselves or to submit the plans to a shop for assembly. □

David C. Schwartz, Cynthia Smith, Mitzi Baker and Mei Hsu are at the Carnegie Institute of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210, USA. For a copy of the blueprints, fill in reader service number 100.

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New lines in labware

A solid scintillator, low-cost spectrophotometer and a new protein/peptide sequencer will be featured at next week's Salon du Laboratoire in Paris, France.

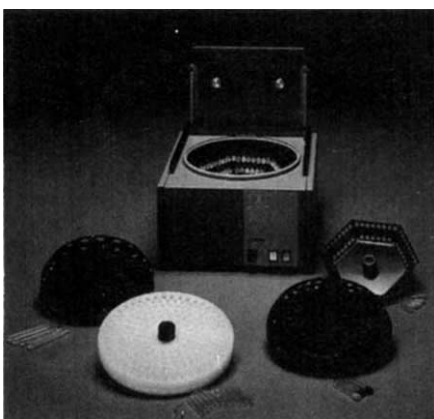
PERSONALIZE sample tubes, colour code diagnostic kits or label hazardous substances with the new **custom-printing service** for laboratory consumables offered by Elkay Products, Inc. (Reader Service No. 101). Test tubes, centrifuge tubes,



Elkay's custom-printing service for lab. consumables.

mailing tubes or specimen containers from 0.5- to 6-inch diameter and up to 9 inches in length can be printed 360° round with, for example, names, addresses, telephone numbers, product name and instructions and volume graduations. There are over 500 colours and a variety of inks to choose from, including autoclavable and write-on ink. Visit Elkay Products on stand 5N050.

Jouan, Inc., exhibiting on stand 5M030, has a new **centrifugal vacuum concentrator** — the Model RC10.10 — which is designed for the rapid concentration of biological and other samples (Reader Service No. 102). The Model RC10.10 can be used to concentrate samples for analysis by HPLC, FPLC, TLC, RIA, EIA, GS/MS and electrophoresis, with virtually 100 per cent recovery, says the company.

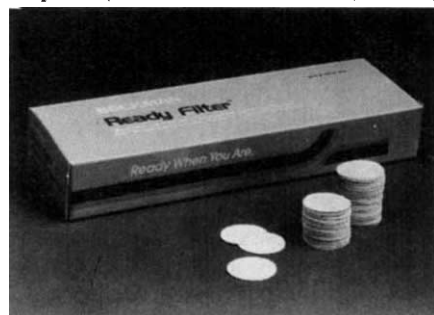


Jouan's centrifugal vacuum concentrator.

Various rotors are available for handling samples from 0.5 to 50 ml, including the high-volume rotor with a capacity of 202 5-ml tubes. Samples are centrifuged at an optimal speed of 1,300 r.p.m., which, the company says, eliminates bumping and foaming, while heat — switchable between ambient and 45 °C — and vacuum are applied to remove solvent. Standard features of the RC10.10 include a corrosion-resistant stainless steel interior, safety glass lid with dual-lid interlock, and automatic vacuum bleeder valve. The RC10.10 can be used with standard high-vacuum systems or can be supplied as part of a concentrator system which includes concentrator, cold trap, vacuum pump and accessories.

Lab. consumables

Beckman Instruments, Inc. is selling a second **solid scintillator** — Ready Filter — designed to cut down on radioactive waste disposal (Reader Service No. 103). Ready

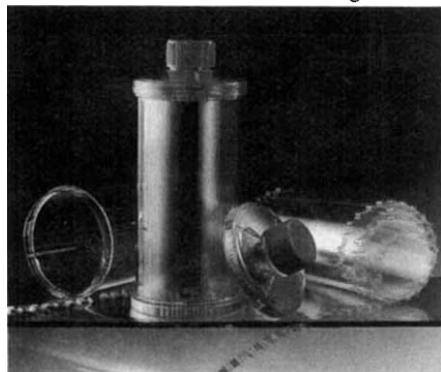


Cut down on radioactive waste with Beckman's solid scintillator.

Filters are glass fibre filter papers coated with Xtalscint which can be used with α , β and γ isotopes. Ready Filters need to be dried and placed Xtalscint-coated side up in the vial prior to counting; this, Beckman says, eliminates the need for scintillator cocktail and allows the filters to be disposed as dry waste. According to the company, when Ready Filters are used in conjunction with the Beckman LS 6000 Series counters, ^3H , ^{14}C , ^{125}I and ^{32}P can be counted with greater than 20%, 70–90%, 50–70% and 70–95% efficiency, respectively. Ready Filters are supplied in boxes or 100 25-mm circles at \$224 (US) for four boxes, or in boxes of 50 strips for cell harvesters for between \$140–385 (US) per box. Beckman Instruments will be on stand 5NR080.

According to Sterilin, higher cell concentrations and cell yields are possible with SPIRA-CEL culture bottles (Reader

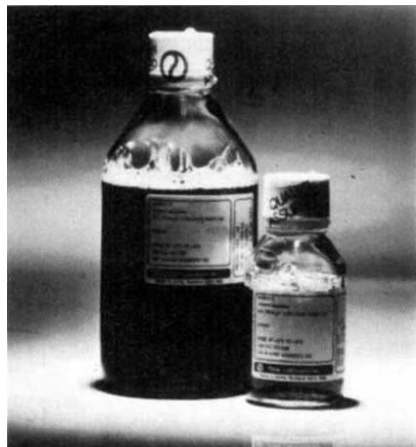
Service No. 104). The bottles offer up to 6,000 cm² of surface area, which, Sterilin says, is seven times greater than that of standard 850 cm² bottles. The unique design comprises an inner polystyrene spiral that has been treated for the optimum growth of anchorage-dependent cells contained within a clear disposable container. The bottles are designed for



Sterilin's SPIRA-CEL bottles designed for increased surface area.

easy access to the spiral for cell harvesting by scraping and have conical bottoms to ensure complete removal of supernatant. Other features include a frosted area for culture identification and fraction rings top and bottom to minimize slippage. Visit Sterilin's wide range of culture conveniences on the Bibby stand, number 5F121.

BioRich 2 is the new serum-free media from Flow Laboratories that is suitable for the culture of a broad range of suspension and adherent cells (Reader Service No. 105). According to Flow, BioRich 2 has been used successfully for monoclonal antibody production, myeloma, hybridoma and lymphoid cell culture, viral production and cell hybridization. The serum-free media is 0.1 µ sterile-filtered and is batch tested for microbial and mycoplasma contamination, growth promotion capabilities, osmolality, pH and endotoxin levels. Flow says that BioRich 2 reduces the risk of contamination, simplifies the purification of cellular



General purpose serum-free culture media from Flow.

products, and is ideal for researchers who want to avoid many of the unwanted proteins found in serum. BioRich 2 comes in 100- and 500-ml quantities for £4 (UK) and £16 (UK), respectively.

J.T. Baker will be exhibiting, on stand number 5M090, their full range of **silica-based media**, including glutaraldehyde-P affinity matrix (Reader Service No. 106). The matrix is produced by bonding a glutaraldehyde group onto the polymer-coated wide-pore silica gel. The company says that the polymer coating leads to less secondary interaction with the matrix and thus higher specificity, and the wide-pore nature of the matrix gives it a high capacity of, for example, 70 mg per gram with Concanavalin A. In addition, the matrix is not subject to swelling and shrinkage with changing salt conditions, says J.T. Baker.

Spectrophotometers

A phase-locked optical system, user-configurable software and the option of add-on application-specific accessories are features of the new Cary UV/visible spectrophotometers from Varian (Reader Service No. 107). The plane-blazed holo-



Cary spectrophotometer: see stand 5F098.

graphic grating (single or double), double-beam optics, and high-performance photomultiplier, offer excellent stray light rejection, automatic dark current correction and a high signal-to-noise ratio, says Varian. Cary spectrophotometers have a wavelength range of 190–900 nm, spectral bandwidth of 0.2–4.0 nm and scanning speed of 0.1–3,000 nm per minute, says the company. Absorbance, % transmission, log absorbance and concentration can be measured as a function of wavelength, time, temperature, distance, or sample number. At the heart of the system is the applications development operating language developed specifically for spectroscopy. This allows instrument, accessory and software control from the IBM PC and the storage of up to 999 methods, data files, reports or keystroke programmes. Prices for the Cary 1 single monochromator and Cary 3 dual monochromator start at \$15,700 (US). Visit Varian on stand 5F098.

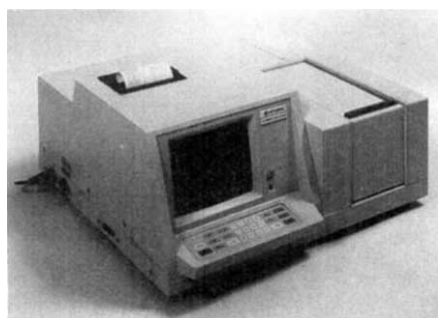
As an upgrade to the PU8700 mouse-driven UV/visible spectrophotometer,



PU8750 Series of UV/vis. spectrophotometers from Philips.

Philips Analytical is offering four new instruments in the PU8750 Series which offer either fixed (2 nm) or variable bandwidths (0.2–2 nm) and either monochrome or colour graphics (Reader Service No. 108). The PU8750's photomultiplier-based optical system and the unique blazed holographic master grating down to 0.2 nm, offers, says Philips, reliable performance over the 0–3 Å range. Four data collection modes are available: absorbance/transmission measured against wavelength, time, concentration and at discrete wavelengths. The latter includes one, two or three wavelength calculations, peak area and height calculations, auto peak search and Bichromatic or Allen corrections for background variance. Features of the data processing software include post-run rescaling, overlay, smoothing, zooming and arithmetic calculation between spectra and constants. Analytical data, routine methods and calibration curves can be stored in the PU8750's non-volatile library facility. Philips will be on stand 5K061.

Hitachi Scientific Instruments' new **all-in-one UV/visible scanning spectrophotometer** — the U-2000 — can be purchased for under £6,000 (UK) (Reader Service No. 109). The U-2000 incorporates a spectrophotometer, screen, keyboard and printer into one compact benchtop unit, and offers a large test compartment to accommodate a variety of samples. The maximum scan speed of 2,400 nm per minute and an autocalibration facility provides fast results, says Hitachi. Menu-driven functions control the various anal-

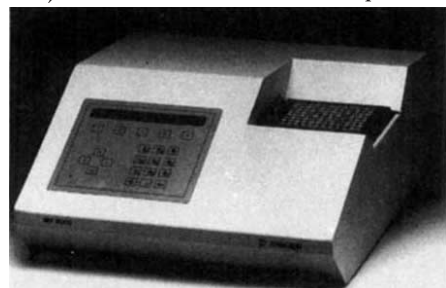


U-200: Hitachi's all-in-one spec.

ysis techniques, which include quantitative calculations, wavelength scanning, time scanning and multi-wavelength measurement. Hitachi Nissei Sangyo will be on stand 5G118.

Measurement means

Visitors to Dynatech Laboratories' stand 5Q032 can see the new range of options available for the MR 5000 ELISA microplate reader, which now includes a **plug-in agglutination module** for large-scale agglutination testing (*Reader Service No. 110*). The MR 5000 can make up to 32



New accessories for the MR 5000.

individual readings taken across each well, with data stored in the reader's memory. The user can set threshold values against control wells so that results are expressed as a simple positive/negative on the 8 × 10-inch printout. The reader can also produce graphic printouts of the percentage of light transmittance of individual wells, columns, rows or whole plates. The MR 5000 comes with an 80-column printer and can be computer-controlled via a RS232 port. To increase the usefulness of the instrument for the purposes of sample identification, Dynatech also offers an optional bar-code reader.

Olympus Optical Co. recommends its Video Micro Scalers for the high-precision **measurement of lengths and distances** of images viewed through a microscope or macro lens (*Reader Service No. 111*). Three models are available in the VMS Series: the \$2,700 (US) VMS 100, the \$3,800 (US) VMS 200 and the \$4,700 (US)



Measure lengths and distances with Olympus' Video Micro Scalers.

VMS 300. The image viewed by the video camera using either a macro lens or a microscope is displayed on the video monitor. Vertical and horizontal lines superimposed on the image can be moved to the required positions by use of the rotary control or an optional joystick. The VMS 100 provides a 4-digit front-panel display of measurements in either metric or imperial units, and the VMS 200 and 300 provide a 5-digit display on both the front panel and the monitor, together with storage of five calibrations for five microscope objectives with different magnifications. The VMS 300 model also has vertical and diagonal line-width measurement and a digital target system for automatic edge detection. The Video Micro Scalers can be seen on stand 5K050.

The model 473A **protein/peptide sequencer** from Applied Biosystems combines Pulsed Liquid or gas-phase chemistry, on-line microbore gradient PTH amino acid analysis with the data handling capabilities of a Macintosh-based data system (*Reader Service No. 112*). According to the company, the integral microbore gradient LC system for amino acid separation and identification provides at least a four-fold increase in sensitivity



Applied Biosystems' model 473A protein/peptide sequencer — see stand 5T141.

over standard-bore HPLC systems. Keeping up-to-date with developments in the field of sample preparation, the Model 473A is compatible with all types of sample supports, including BioBrencoated glass fibre filters, solid-phase supports, PVDF, ProBlott and ProGlas. The variable injector system allows up to 100 per cent of the sample to be analysed, enhancing the system's sensitivity, says Applied Biosystems. The Macintosh-based data system provides the user with options for baseline and 'lag' correction, chromatographic noise filtering and peak integration. Taking advantage of the graphics and desktop publishing capabilities of the Macintosh, outputs can be customized into manuscripts and reports.

Under the microscope

Planar Biomed introduces the CM-3 **cryo-microscope system** which allows direct observation of the critical changes that occur within cells, tissues and other



CR-3 cryo microscope system.

samples during freezing and thawing (*Reader Service No. 113*). The system comprises cryo-stage, heat exchanger, dedicated microcomputer, electronic interface unit and control software. The cryo-stage can be fixed to the x-y table of most microscopes. Its conductive design provides a minimal light path across the viewing area, allowing the use of short working distances and high resolution, says the company. Samples of up to 10 µl are placed on the optical glass heater slides, which are fitted with a T-thermocouple for monitoring sample temperature. The cryo-stage can be maintained at constant temperature or the user can pre-programme up to 12 temperature gradients and 'hold' intervals. The sample temperature range can be between +100 °C and -100 °C, and both sample and stage temperature are displayed on the electronic interface unit. The optical data is typically recorded on video for subsequent image analysis. Menu-driven software allows the user to calibrate, enter program parameters and control operations. Planar Biomed will be on the Flobio stand, number 5M010.

The facility for **automatic remote-controlled microscopy** with Nikon's Microphot FXA microscope is now available (*Reader Service No. 114*). The Microphot FXA can be controlled by a PC, or can be linked up to a modem or serial printer via the RS232C port. Menu selections such as auto bracketing, film format and quantitative photometry are chosen using the slide-out keyboard and are displayed on the 40-character, 8-line LCD. Special features of the FXA include a motorized condenser that automatically sets the aperture according to the objective in use, a motorized revolving quintuple nosepiece, and autofocusing with objectives between 1–20 × for photomicrography at low magnifications. Data that is to be imprinted on the film is entered via the keyboard. Nikon will be demonstrating their full range of microscopes on stand 5N028. □

These notes are compiled by Diane Gershon from information provided by the manufacturer. To obtain additional information about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.